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INTERSTITIAL CELL SEEDING AND DYNAMIC CONDITIONING OF AORTIC HEART VALVE SCAFFOLDS

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INTERSTITIAL CELL SEEDING AND DYNAMIC CONDITIONING OF AORTIC HEART VALVE SCAFFOLDS

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ABSTRACT

In 2011, U.S. Markets for Heart Valve Repair and Replacement Products estimated that roughly four million people in the United States are diagnosed with a heart valve disorder annually. And in the following year, the American Heart Association reported that approximately 35,000 deaths in the US were either directly or indirectly attributed to heart valve disease.

Diseases of the heart valve are degenerative in nature and therefore progressively worsen unbeknownst to the patient until symptoms become clinically relevant. By this time, the valve is in such poor condition that complete replacement is often the only effective treatment. Current solutions are mechanical and bioprosthetic replacements, but each is associated with its own inadequacies. As a result, science is now looking to the promising field of tissue engineering for solutions to the limitations of current valve replacement options. A patient-tailored, translational approach to heart valve replacement that is safe and effective will expectantly be the result of efforts put into this rapidly growing area of research.

Present experiments hypothesize that the success of tissue engineered heart valves lies in the complete recellularization of a scaffold with autologous cells prior to implantation. While external cell seeding of the valve to avoid thrombogenicity seems to be the most obvious need, additional focus on interstitial recellularization is necessary to obtain a truly biocompatible replacement capable of valve remodeling and regeneration. Our short term goal is to obtain a fully recellularized, viable valve scaffold. Thus far, infiltration of externally seeded cells into the acellular tissue scaffold has been shown to some extent, but has led to many questions concerning the effectiveness of those seeding methods. More specifically, there are concerns about the characterization of the cell phenotypes found in the interstices of the cusp and their effect on the success of the entire valve construct in vivo.

The following document proposes a novel method of cell injection into acellular aortic heart valve cusp scaffolds followed by mechanical conditioning as a prerequisite to complete valve recellularization that will yield proper valvular interstitial cell phenotype within the construct. Present studies have optimized the cellular injection technique, determined appropriate conditioning methods, and shown cell retention and differentiation within the interstices of the cusp.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 The Impact of Cardiovascular (Valvular) Disease

In 2011 it was estimated that roughly four million people in the United States were diagnosed with a heart valve disorder annually [1]. The prevalence of diseases associated with heart valves is astounding and means good business for those in manufacturing. As "the global leader in the science of heart valve and hemodynamic monitoring," Edwards Lifesciences reported impressive sales during the 2012 fiscal year so much so that they are projected their 2013 sales of Surgical Heart Valve Therapy Devices to be more than $800 million [3].

Often the only solution when treating degeneration or calcification of heart valves is replacement of the entire valve. Current mechanical and bioprosthetic options for heart valve replacement are mildly satisfactory; the real promise for complete success in the future of replacements lies with tissue engineering. There is much potential in this rapidly advancing field, and these efforts continue to be highly funded and researched. Thanks to a surge of interest in cardiovascular tissue engineering within recent years, major accomplishments in the field have been recorded and are continuously being built upon by those who follow.

1.2 Relevant Anatomy

There is no doubt that the aortic valve is the most stressed tissue in the human body, completing its open/close cycle over three billion times in a person's lifetime [5]. With approximately 7200 liters of blood passing through its opening during the course of a single day, the aortic valve experiences great shear flow stresses and tremendous amounts of pressure compared to the other three valves of the heart [6]. The output that is necessary for normal functioning of the aortic

valve is incredible; therefore the structure and tissue that the valve is composed of must be able to withstand these mechanical requirements.

The aortic heart valve has three cusps that form semilunar attachments to the aortic root, which is continuous with the structure known as the aortic arch [7]. The aortic annulus is the 'ring-like' structure that is formed circumferentially around the root as a result of the cusp attachments. These structures all provide stabilization for the delicate and thin cusp tissue that is made mostly of collagen, elastin, and GAGs.

The cusp is comprised of three distinct layers: the fibrosa, spongiosa, and ventricularis as seen in figure 1. The collagen and elastin components give the cusp its load-bearing properties and resiliency, respectively. The cusp's ability to repeatedly be forced out of shape to open allowing blood to flow out of the heart and then return to its closed position to prevent the backflow of blood into the heart is facilitated by these matrix components. It is the glycosaminoglycan (GAG) inner layer of the cusp that allows for the easy, almost frictionless changes in conformation of the valve. This layer acts as the shock absorber to help the valve withstand the constant differences in pressure and impact loads [5].

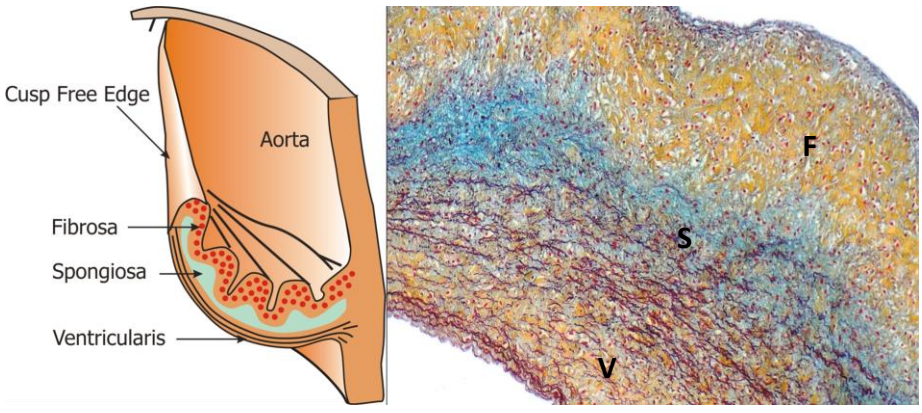


Figure 1: Aortic Heart Valve Cusp Structure
Physiological arrangement of cusp layers [8] (left), Movat's Pentachrome Stain of a fresh (native) cusp [9]: nuclei stain red, collagen stains yellow, elastin stains deep purple to black, GAGs stain blue (right)

1.3 Heart Valve Disease Etiology & Pathology

Heart valve disease can be caused by general wear of the tissue, an inflammatory response that is initiated by cells in the area, bacterial infections, congenital defects, or other idiopathic origins. When analyzing the stresses experienced by the aortic valve, general wear of the valve seems to be an obvious cause of degeneration, especially as the world population is living longer. It is also understood that the flow of blood through the valve can cause micro-tears in the tissue; these tears trigger an inflammatory response by the native valvular cells. Valve destruction can also be caused by severe cases of rheumatic disease; endocarditis (inflammation of the endocardium) is the result and it leads to scarring and damage of the valve leaflets, once again initiating an inflammatory response. This particular cause is overwhelmingly prevalent in developing countries and indigenous groups within the developed world [10]. Many of the other causes of heart valve disease and degeneration are not fully understood, but solutions must be ready to combat them nonetheless.

Heart valve disease causes the failure of various mechanisms of the valve that must be retained in order to provide efficient function. Two of the most prevalent malfunctions are insufficiency (also referred to as regurgitation) and stenosis. Regurgitation is described as the improper closing of the valve which then causes blood to flow backwards through the aortic valve and back into the heart. Stenosis is classified as the hardening of the valve due to calcium build-up (calcification). These defects in the function of the aortic valve can be the cause of other pathologies such as left ventricular hypertrophy. This condition of the heart develops due to decreasing insufficiency in valve function. The heart must compensate for this decrease in cardiac output by pumping harder causing the left ventricle to pump harder and therefore increase in size (like any muscle would

when it is over worked). Eventually, if left undiagnosed and untreated, this series of events will lead to heart failure and death [11].

1.4 Current Treatments

As previously mentioned, by the time a patient begins noticing symptoms of valve deficiency it is after the valve has already undergone major degenerative processes leaving it inadequate to satisfy the great requirements of the heart's total cardiac output. For any pathology that might be present there are no effective drug treatments that can be used [5]. In most cases, alternative measures are not even considered; doctors opt for the patient to receive a valve replacement. As one of the short-term measures that can be used to temporarily relieve the symptoms of valve disease, the doctor may suggest balloon valvuloplasty. This procedure is typically regarded as a bridge-to-aortic valve replacement and not an end point treatment for any patient.

A revolutionary new method of heart valve replacement is gaining fast support among all those in the medical field. The technique is called Transcatheter Aortic Valve Replacement or TAVR. The procedure is innovative because it employs a minimally invasive approach to the replacement of a large heart structure. Catheterization is used to insert the non-expanded valve into the femoral artery where it is then maneuvered through the vasculature until finally reaching the opening of the aortic valve. The replacement valve is forced through the pathologically stenosed valve and then deployed by balloon expansion of the outer stent covering. The expansion of the stent forces open the stenotic valve, the new valve then secures itself in place by relying on the hard calcium deposits to act as anchors. The patient does not need to be put on heart-lung bypass in order to replace the valve. (Note: There is also another method to performing this surgery that is referred to as transapical, meaning through the apex of the heart.) Currently though, TAVR is only considered for those patients who are deemed a 'high-risk' for surgery and for patients with

calcific stenosis. Other valve pathologies would not provide the solid anchoring that is present on the calcified valve [7].

The most widely used treatment option for heart valve disease is still an Aortic Valve Replacement (AVR). This involves the complete removal of the pathologic valve and replacement with the new valve. As discussed previously, left ventricular hypertrophy is a result of valvular disease; after replacement of the dysfunctional valve the tissue can actually revert back to a physiologically appropriate mass. This is one of the most significant and obvious signs of effective treatment [5]. Following a valve replacement most patients notice quick improvements on their quality of life.

After a patient has been diagnosed with severe heart valve disease and has been approved for AVR it then becomes a discussion of what type of valve will be used. There are mechanical and bioprosthetic options that are currently on the market. No matter which valve option a patient/doctor chooses there is about a 10-15 year range that the valve is guaranteed to last. The goal of all major heart valve manufacturers is to improve the long-term durability of the valves so that patients would not have to undergo several surgeries throughout their lifetime [12].

1.4.1 Mechanical Heart Valve Replacements

The first designs of replacement heart valves were for mechanical models, with major breakthroughs occurring in the 1950's to 60's; researchers made many developments in the field of science and engineering during this time and applied

Figure 2: Mechanical Heart Valves
Starr-Edwards Silastic Ball Valve (left) and St. Jude Medical Masters HP Valve (right).

them effectively to the clinical need for heart solutions. The ball-in-cage design prevailed for many years until the tilting disc's emergence. Eventually the pyrolytic carbon bileaflet valve was designed in the 1970's and became the 'gold standard' for mechanical valve replacement. Since St. Jude Medical introduced this pyrolytic carbon valve much of the innovation in this area has come to a halt; only minor features of the mechanical valves have evolved over the last forty years.

Some of the early challenges with mechanical valves were related to closing load, material fatigue, and cavitation. Transvalvular pressure produced variations in the load experienced by the valve which lead to "impact wear" and "friction wear." These two forces worked to weaken the structure of the valve. Also, like any device, material fatigue played a large role in the valve's success or failure. This obstacle was overcome with the evolution of materials; once the switch from metal to pyrolytic carbon was made this seemed to solve fatigue issues. The third most significant failure mode was cavitation, which is characterized by the formation of microbubbles in the blood. The microbubbles can, over time, cause pitting and erosion on the surface of the valve. This problem was solved during the 1970's and 80's with small engineering design changes; since then, no reports of failure of the mechanical valves have been reported around those modes of mechanical failure.

Although mechanical valves are the most durable solution (typically said to last for 20-30 years) for heart valve replacements there is still one very large issue that has yet to be completely avoided. The thrombogenicity of the artificial surface is cause for much concern. Not only are the devices non-biological, they also introduce turbulent flow regimes in the flow of blood. The turbulence can activate platelets which in turn initiate the formation of thrombi. To prevent this process from occurring, patients are put on life-long anticoagulant therapy. This involves the

administration of Warfarin, a drug that minimizes the risk of thrombus formation on the valve surface. Because this drug decreases the blood's ability to coagulate on the valve it also means that it prevents coagulation systemically leaving the patient vulnerable to major bleeding events. This reliance upon the close monitoring of a potentially dangerous and expensive drug therapy is the main factor that has kept mechanical valve replacement out of developing countries [13].

1.4.2 Bioprosthetic Heart Valve Replacements

Figure 3: Bioprosthetic Heart Valves Medtronic Freestyle Tissue Valve (left) and Edwards Lifesciences Perimount Magna

The solution to the possibility of thrombogenicity with mechanical valves was solved with the advent of bioprosthetic valve (BPV) technology. These valves are made from either porcine aortic valves or bovine pericardium that has been chemically fixed, cross-linking the tissue and masking the antigens present in the xenogeneic materials. There is much debate between the tissue options and their design features, but thus far most options have shown comparable results. Although patients do not have to be on any drug therapy for proper functioning of the bioprosthetic valve, their longevity is questionable due to other degenerative processes, such as calcification and general wear and tear [13]. BPVs are predicted to last 10-15 years which is a much lower expectation than that of the mechanical valve [14].

The xenogeneic tissues are fixed with a dialdehyde known as glutaraldehyde. This process was thought to completely cross-link molecules of the ECM rendering the tissue anti-thrombogenic and inhibiting calcification. This however, was not necessarily the case because BPVs were shown to be highly calcified upon explantation and the presence of inflammatory cells was seen

on not only the surface of the valve but also infiltrating into the tissue. Some of the issues with inflammation may be due to the tissue still eliciting an immunogenic response by the host. During the fixation process, if there is insufficient masking of the receptors or glycoproteins in the tissue the components would inevitably cause immune response. In more recent years it has been determined that higher concentrations of glutaraldehyde can be used to come as close to ensuring a complete cross-linking as possible. Researchers then looked into the effects of the higher concentration of glutaraldehyde used. The numerous unbound glutaraldehyde molecules that were present needed to be extracted because of their tendency to be pro-calcific. By removing these free dialdehydes the tissue was made available for the formation of an endothelial layer to flourish on the surface of the valve and calcification initiation was delayed [13].

The processing of the valves needed to be assessed to find an answer concerning durability after the tissue was fixed. The cross-linking of the tissue obviously had an effect on the overall strength and mechanical properties of the valve; the valve must rely purely on the integrity of the ECM components with no active remodeling occurring when there is valve damage or degradation [14]. When the glutaraldehyde cross-links the tissue, the GAG-rich spongiosa layer of the cusp loses its ability to reduce the stress throughout the cusp during the open/close cycle. With this layer virtually non-functional, the valve begins to wear at a faster rate. The annulus of the valve was also affected by fixation because it made the tissue much more ridge. The annulus is supposed to be able to dilate as the heart pumps and the valve opens and closes. After the tissue in the annulus is cross-linked it loses most of its ability to function properly causing more stress on the cusps that are attached to it.

In a recent Veterans Affairs Study by Mancini et al. a comparison was made of BPVs and mechanical valves that looked specifically at mortality and reoperation rates. After 15 years,

follow-ups were conducted and it was found that all-cause mortality was lower for the patients implanted with the mechanical valves than the BPVs. Also, the reoperation rate was much lower for mechanical than BPV. The data all pointed to what some had already concluded, which is that as far as long-term durability is concerned the mechanical valve is the superior [15].

Although the mechanical valve is more durable and can be projected to last longer, the BPV is still the best choice for those patients who cannot be put on anticoagulant regimes. This group would include women who are pregnant or are planning to have children and those who may not want to be put on drug therapies due to a very active lifestyle. There is a trend of patient populations that receive certain valves, such as elderly patients (65 years and older) typically receive BPVs because of the reduced chance for calcification, and younger patients/children receive mechanical valves due to the decreased number of reoperations required [5].

1.5 Tissue Engineering Solutions for Heart Valve Replacements

Tissue Engineering seeks to make curative solutions for patients who are seeking long-term treatment of disease and tissue degeneration. The constructs that are being researched and tested are not simply there to try to compensate for the damaged tissue, the aim is create an actual living tissue that can be implanted into a human that will, from that point on, grow and remodel. There are five main approaches to heart valve tissue engineering which have been seen as the main focus in more recent research: 1) forming a tissue through in vitro, bioreactor conditioning by seeding cells on some type of biodegradable scaffold; 2) seeding cells on natural biodegradable scaffolds; 3) implanting degradable tissues that are to be remodeled by native cells (also referred to as guided tissue regeneration); 4) implanting a decellularized valve scaffold; 5) processes that aim to generate tissues in vivo through native pathophysiological conditions [16].

The focus of the current project is in approach 1 as listed above. Referencing the Tissue Engineering Paradigm found in figure 4, the first step is to consider is the appropriate cell type with which to seed the scaffold. Cells can either be obtained from fully differentiated sources or stem cell populations. The possibilities for research using embryonic are limited due to government policy, ethical debates, and social stigmas. This is why investigation into alternative sources has led to great promise in adult stem cells. Several of the cell sources that are promising are vascular-derived cells, bone marrow-derived cells, umbilical cord-derived cells, and adipose-derived stem cells [17]. Most of these cell sources have been shown to differentiate into many cell types, making them pluripotent and useful for the application of tissue engineering.

Next is the need to develop an appropriate scaffold that will support either cells seeded externally and internally or cells that infiltrate from the patient's surrounding native tissues. The scaffold must also be able to perform mechanically in order to withstand the tremendous number of cycles that it is to be put through once implanted into the body. Many different types of scaffolds have been researched and tested, including, allogeneic (human) or xenogeneic (animal) tissues. These are constructs made up of ECM components or are polymer-based structures. When using human and animal tissues, a process called decellularization is employed to remove the native cells from the valve. This is a process that involves a series of detergent washes that 'clean out' the cells, leaving only the ECM components (i.e. collagen, elastin, laminin, etc.). These techniques must be able to completely remove all immunogenic materials, and it must also be reproducible.

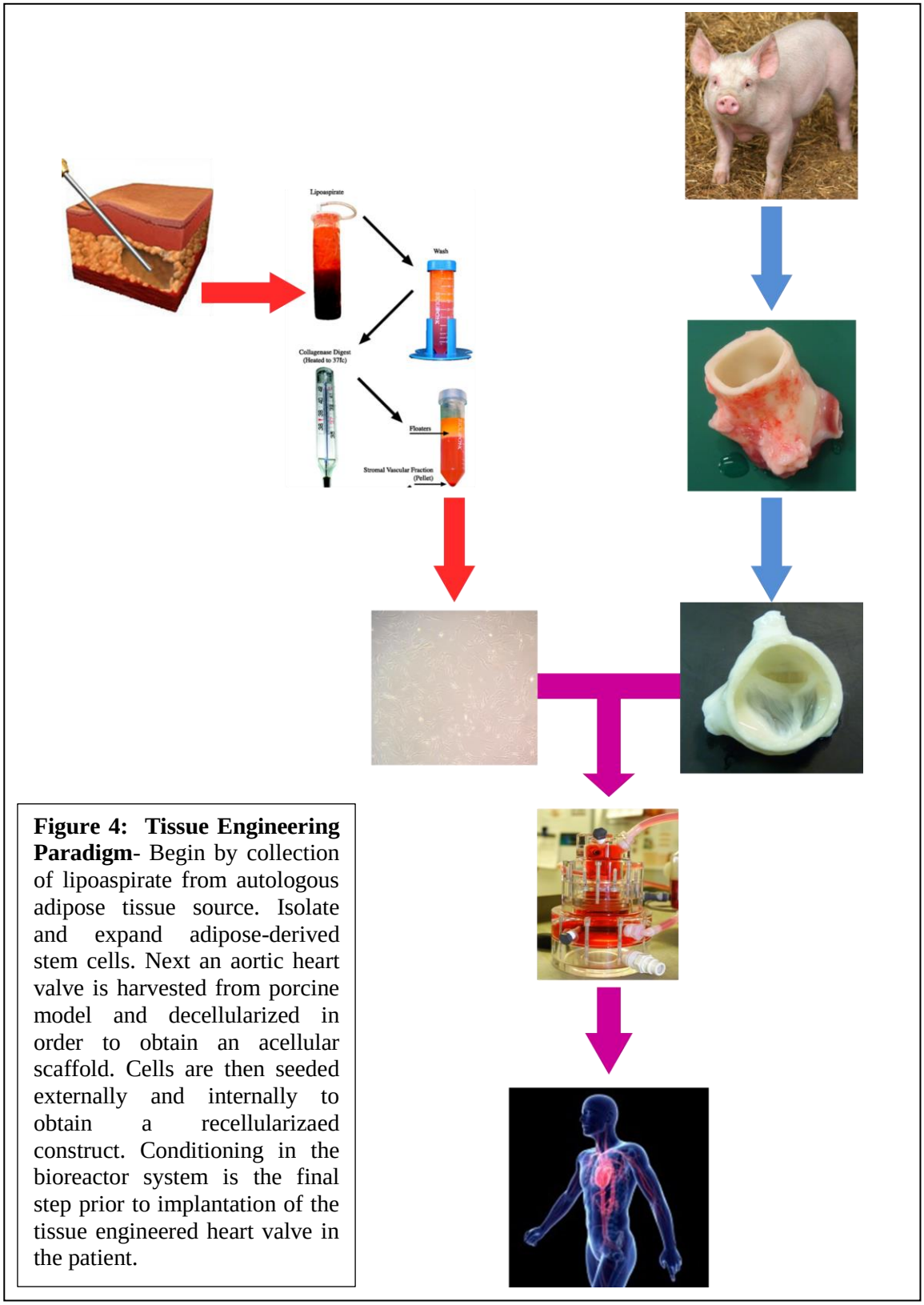


Figure 4: Tissue Engineering Paradigm- Begin by collection of lipoaspirate from autologous adipose tissue source. Isolate and expand adipose-derived stem cells. Next an aortic heart valve is harvested from porcine model and decellularized in order to obtain an acellular scaffold. Cells are then seeded externally and internally to obtain a recellularizaed construct. Conditioning in the bioreactor system is the final step prior to implantation of the tissue engineered heart valve in the patient.

In regards to decellularization, initial data collected by Cryolife, Inc. during in vivo animal studies was very promising; the studies were performed using the Synergraft™ tissue engineered heart valve. The scaffolds were decellularized porcine aortic heart valves that were implanted into sheep in the pulmonary valve position for 150 days. When the valves were explanted the researchers observed great host-cell infiltration; valves were repopulated by fibroblast-like cells. They also saw that there was no increase in the calcium content of the valve afterwards. The research proceeded to clinical trials based on their promising data. Four of the Synergraft™ valves were implanted into children in the pulmonary position. All seemed well at first, the surgery went well and recovery was typical, but after six weeks the first patient died due to sudden cardiac arrest. The second patient died one year post-op, again due to sudden cardiac arrest. The third patient died within the 7th day of implantation due to the rupture of the graft. And lastly, the fourth patient was ordered to have the valve explanted after only two days of implantation. Upon review of the explanted valves it was found that the tissues showed extreme amounts of inflammation and the cusps were practically non-existent [18].

It was later concluded that the valves had not been completely decellularized; there were porcine cells still present within the scaffold. This study cast a dark shadow over tissue engineering that is still seen today. It did, however, shed light on areas that needed to be improved. Standards need to be established in which a valve can be said to be 100% decellularized so that catastrophic failures like those in this study never happen again. Also, this study showed that not all animal models are applicable and appropriate to model in vivo conditions of a human, so we must also work to discover the best animals for implantation.

The final stage in the tissue engineering paradigm before implantation in the patient is the conditioning of the tissue construct in a bioreactor. Such conditioning devices can be seen

throughout cardiovascular research; systems have been used for vascular grafts, myocardial replacement tissues, and heart valves. The use of bioreactor tissue conditioning has been shown to aid in the development of mechanical strength within the construct and modulate cellular function [19]. Refer to section 2.5 for more on mechanotransduction during pre-conditioning of valve constructs seeded with cells.

Moving forward, many realize that innovation in most areas of tissue engineering is coming from academia and start-up companies [13]. The valves that are currently available for patient implantation are 'just OK,' not great. It is tissue engineering that offers truly innovative and revolutionary solutions for heart valve replacement. There are challenges (reference Appendix A) that will arise from clinical translation of heart valve tissue engineering, but many researchers have already begun the process of directing their research toward combating them.

CHAPTER 2: HEART VALVE TISSUE ENGINEERING, INTERSTITIAL SEEDING, AND CELLS

2.1 Heart Valve Tissue Engineering: A Shift in Focus

Heart valve tissue engineering solutions have seen a shift to putting more of an emphasis on pediatric applications [16, 19-24]. This may be the first clinical application in which tissue engineering replacements are introduced into patients due to the fact that it is the younger population who would benefit the most from a construct that is able to grow and remodel as they age. Current mechanical and bioprosthetic replacements are assumed to be sufficient for the aging population, but neither of these options is reasonable for use in children who would quickly outgrow them. In preparation for clinical translation of tissue engineered solutions researchers are attempting to shift their research toward materials and methods that would be more easily transitioned to use in patients (from section 1.5 and Appendix A).

Highlighting some of these proactive steps toward clinical translation, more focus has been placed on the concerns surrounding patient variation and an effort has been made to determine ways in which tissue engineering solutions can adapt to, detect, and combat these differences. From patient-to-patient, large variations are seen in the way each person responds to wound healing and tissue remodeling. The overall success of a replacement relies on the ability of the patient's own biological processes to aid in the healing and remodeling of the tissue. Research into methods of controlling this variability will be fruitful toward the acceptance of the use of tissue engineered replacements and proof of efficacy amongst all patient populations.

The next step toward clinical translation is addressing the set of safety, efficacy, and quality control standards by which tissue engineered devices will need to be tested and validated. Beginning with safety studies performed in vitro, a standardized bioreactor testing system will

need to be established which will be a large undertaking in itself. The tissue engineered solutions will also need to undergo performance and functional testing protocols that need to be created and agreed upon. And lastly, in vivo preclinical studies need to be designed in such a way that they address actual human response to the device. Currently ovine and porcine models are considered the most robust in vivo model for heart valve testing. Sheep are generally helpful to use in calcification prediction and pigs in thrombogenicity potential [20]. But neither model is reliable for comparison in immunological response of the host. Also, the ovine cardiovascular system is able to heal more quickly and completely than a human's so it is difficult to understand the wound healing process of the replacement valve fully [16]. Researchers however, do not see these concerns and challenges as a deterrent to continuing progress toward the end goal of developing a tissue engineered replacement that will undoubtedly revolutionize modern medicine.

2.2 Previous Interstitial Cellular Seeding/Infiltration Techniques and Outcomes

Many methods for cell seeding have been tested and reported in literature. One of the more commonly seen methods uses decellularized valve scaffolds that are externally seeded with cells and dynamically conditioned in vitro with the hope of observing cell infiltration. In Schenke-Layland et al., cells collected from ovine sources (endothelial cells and myofibroblasts) were externally seeded onto decellularized porcine pulmonary valves that were then subjected to a maximum of 16 days in a pulsatile bioreactor system. Cell infiltration into the cusps was observed after the conditioning period. It would seem as though the myofibroblasts (MF) had in fact been able to migrate into the cusp and repopulate the structure. Since the MF was used to seed, the cells in the interstices stained positively for both Vimentin and α-SMA [24-25]. When discussing the results of the study the researchers admitted that a less invasive approach for cell isolation was needed and that they were not sure whether or not the high levels of α-SMA were an

indicator of positive tissue remodeling or if it would lead to the beginning of an unfavorable pathological response.

Other groups have developed more novel techniques of infiltration. Cushing et al. reported on the method of serum deprivation in order to repopulate an acellular scaffold. They isolated valvular interstitial cells (VICs) from porcine aortic cusps and seeded those externally onto acellular porcine aortic cusps. The protocol called for recurrent exposure of seeded cells to serum deprivation (using media with 5% FBS instead of 15% FBS) [26]. Although they did observe an 'accelerated' cell infiltration, as opposed to infiltration when using a normal serum level, the process was still lengthy. Infiltration began around 2 weeks, but the scaffolds were not considered fully repopulated until 4 weeks. While the result of obtaining VIC recellularization of the cusps is a great outcome, it is one that is not clinically relevant due to the use of an inaccessible cell source and the time in which it took to achieve. Another note to be made is that during the 4 weeks that the study was conducted, the cusps were in no way mechanically stimulated or dynamically conditioned.

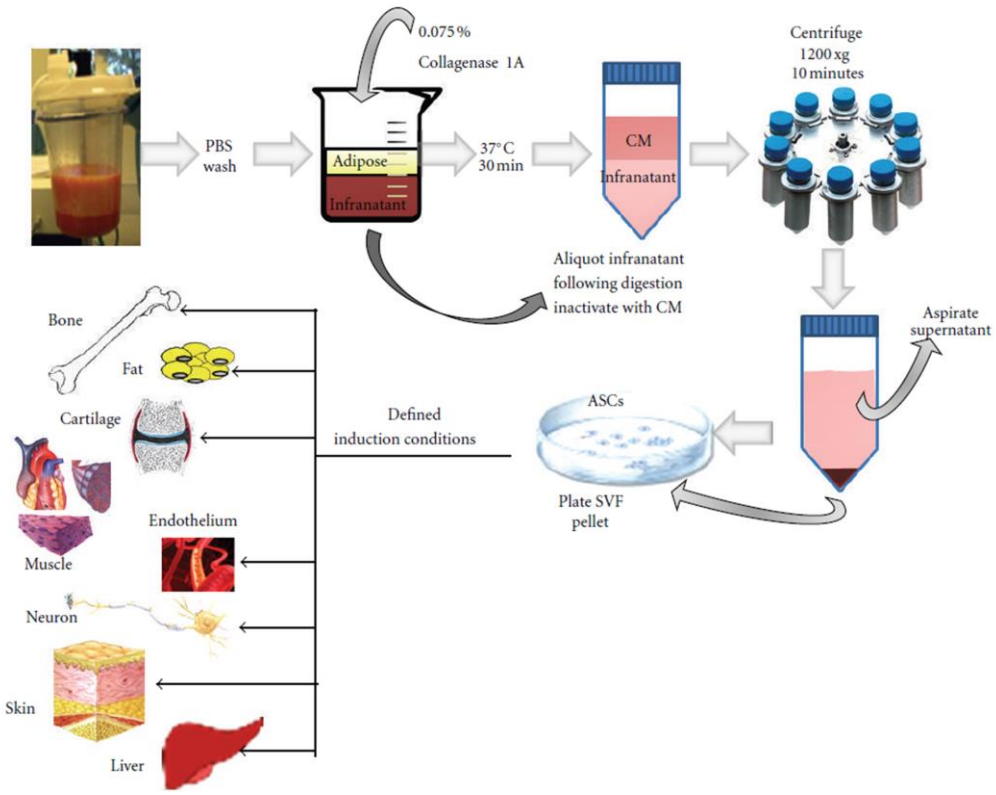
Another novel approach to heart valve scaffold seeding is coined the "self-seeding heart valve." The technique developed by Jordan et al. uses antibody surface conjugation as a means to attract circulating progenitor cells in vivo to repopulate the scaffold. The group used CD133 antibodies that were conjugated to decellularized porcine pulmonary valves. The conjugated valves were then transplanted into sheep in the pulmonary position and explanted after 1 and 3 months. Again, repopulation of the cusps was observed, but expression of α-SMA was found throughout the cusp even after 3 months of implantation [27]. They do not seem to believe that the extensive expression of α-SMA will cause any later issues, but as described in the following section, 2.4, α-SMA may not be what researchers want to see throughout their cusps. Also, no immunological

stains were reported; the cells that are staining within the cusp structure could be macrophages or other lymphocytes that have infiltrated.

2.3 Adipose-Derived Stem Cells

When seeding tissue engineered scaffolds for regenerative medicine the challenge of stem cell availability can be one of the most difficult to overcome. Both scientists and clinicians are looking for cell sources that are in line with the criteria listed by Gimble et al.: 1) can be found in abundant quantities; 2) can be harvested by minimally invasive procedures; 3) can differentiate along multiple cell lineage pathways in a regulatable and reproducible manner; 4) can be safely and effectively transplanted to either an autologous or allogeneic host; 5) can be manufactured in accordance with current Good Manufacturing Practice guidelines [28]. These requirements are necessary in order to develop clinically translatable approaches to tissue engineering. Many cell sources are ruled out due to very small yield from harvesting, such as bone marrow-derived mesenchymal stem cells; out of the $6x10^6$ cells collected it is believed that only 0.001-.01% are actually stem cells [29]. Or they are excluded because of the invasive and painful procedures used to obtain them. For example, bone marrow collection is an extremely painful procedure and one that most patients would not choose if there were a better option. These and many others are reasons in which the focus has shifted to investigation into adipose-derived stem cells (ADSCs). They are readily available and easy to obtain through routine liposuction procedure. The process of ADSC collection, isolation, expansion, and differentiation is displayed in figure 5. Out of approximately 1 gram of adipose tissue $2x10^6$ cells can be collected and 10% of those are believed to be stem cells [29]. ADSCs are also known to differentiate into a large variety of cell lineages, as seen in figure 5.

Figure 5: Collection, Isolation, Expansion, and Differentiation of Adipose-Derived Stem Cells



Lipoaspirate is first collected from the patient, and then collagenase is added in order to enzymatically digest the tissue. Centrifugation of the infranatant isolates the stromal vascular fraction (SVF) from the supernatant. The ADSCs can then be plated and expanded. ADSCs have been shown to differentiate into many cell types [29].

2.4 Valvular Interstitial Cells

Within in the last decade much has been hypothesized and/or discovered about valvular interstitial cells (VICs) and their function within the heart valve cusps. The valvular interstitial cell is the most prevalent cell type in the heart valve; the heart valve is also the only location in the body in which VICs are found. They function to maintain valve structure and they aid in repair and remodeling of the cusp microstructure. The repair function of the VIC is only initiated after some type of trauma or exposure of the cusps to abnormal flow.

Avrum Gotlieb's group has proposed one of the more popular opinions on VICs. They claim that VIC phenotypes can be organized into five groups according to their function: embryonic progenitor endothelial/mesenchymal cells, quiescent (qVIC), activated (aVIC), progenitor (pVIC), and osteoblastic (obVIC). qVICs are the cells that are found in the adult valve that participate in the maintenance of normal valve physiology; they are believed to maintain the characteristic avascularity of the cusp structure by inhibiting angiogenesis. aVICs regulate pathological responses that occur in valve disease and injury. pVICs are believed to be a

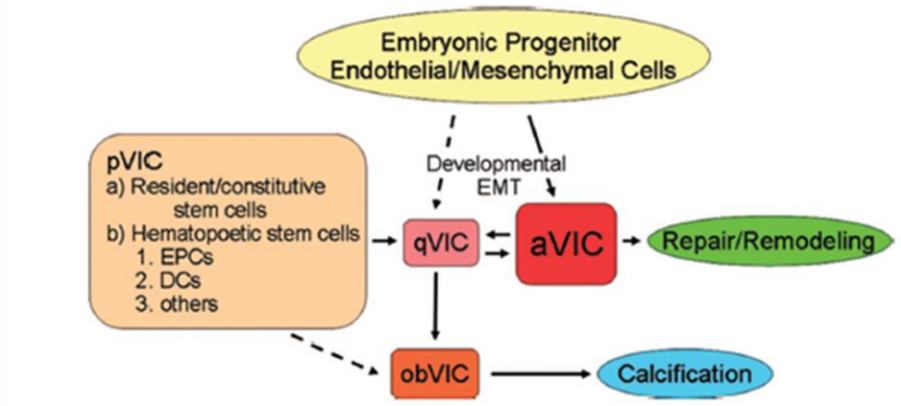


Figure 6: Valvular Interstitial Cell Phenotypes – Solid arrows depict the transitions that are currently understood. Hatched arrows depict possible transitions for which there is currently no solid evidence [28].

population of cells that play an important role in valve repair. obVICs are the cells that are present in calcific valve pathologies; they regulate chondrogenesis and osteogenesis [28].

"The pathogenesis of cardiac valve disease correlates with the emergence of muscle-like fibroblasts (myofibroblasts)." This quote from Walker et al. uses the word myofibroblast, but in accordance with the previous description of VIC phenotypes, this cell type can also be termed an aVIC (activated VIC). In all pathologies of the heart valve, for example, degeneration and calcification, aVICs are found throughout the tissue. And it is α-SMA staining that is the hallmark marker to confirm aVIC presence. The results from studies described in section 2.2 and many others that report extensive staining of α-SMA in their tissue engineered valves are worrisome due to the pathological consequences that could and would be sure to follow with prolonged expression of α-SMA. Many researchers in heart valve tissue engineering support the understanding that activated VICs are not ideal to have within a cusp that is implanted within a patient [30-37].

The status of VIC activation is dependent upon the biochemical and biophysical cues within their microenvironment [36]. In vitro, molecules such as TGF-β have been shown to induce and significantly increase VIC expression of α-SMA and other aVIC markers. A synergistic relationship in cell expression of α-SMA is seen when TGF-β exposure is coupled with mechanical stress on the cells [37]. In vivo, TGF-β is secreted by VICs in response to injury and inflammation of the tissue and therefor leads to the increased presence of aVICs. This mechanism is important when considering therapies that can decrease or even reverse pathological activation of valvular disease. It is the goal of tissue engineering to design around these two parameters to provide conditioning and molecular signaling that is similar to native cusps in order to attain a valve that is ready for remodeling and regeneration in vivo.

2.5 Mechanotransduction and the Effect of ECM on Stem Cell Differentiation

The use of bioreactor systems to pre-condition cell-seeded constructs is typically seen in most tissue engineering approaches. These systems are used to expose seeded cells to varying mechanical forces to induce cellular mechanotransduction. When looking specifically at heart valves and the valvular interstitial cells within the tissue it is important to mimic the unique mechanical environment at this site. Several studies that aimed to recreate the native heart valve tissue mechanics have confirmed that applying cyclical mechanical stimulation of cells leads to higher rates of cell proliferation, enhanced production of ECM components, and differentiation of stem cells to a desired phenotype in vitro [38-40].

Mechanical stimulation combined with the presence of extracellular matrix components, such as collagen and elastin, encourages a synergistic relationship on improved stem cell differentiation and scaffold remodeling. Following the decellularization process ECM components and specific binding sites are retained. These features of the scaffold allow for better cell adhesion through integrin attachment and signaling to encourage phenotypic changes. The acellular scaffold is able to provide a unique niche in which cells can attach, differentiate, proliferate, and lay down extracellular matrix. It is therefore the ideal biomaterial to use for tissue engineered heart valves due to its ability to induce stem cell differentiation and remodeling of the structure [41].

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

Tissue Decellularization

0.05M NaOH, (0.05% SDS-sodium dodecyl sulfate, 0.5% Triton X-100, 0.5% Na-Deoxycholate, and 0.2% EDTA, in 10mM TRIS, at pH 7.5) (all chemicals were of the highest purity available and obtained from Sigma-Aldrich Corp., Lakewood, NJ)

DNase – deoxyribonuclease (Sigma, Steinhelm, Germany)

RNase – ribonuclease (Thermo Fisher Scientific, Waltham, MA)

High purity 1,2,3,4,6-Penta-*O*-galloyl-beta-D-glucose (penta-galloyl glucose, PGG) was a generous gift from N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium (www.omnichem.be)

Neutralization

Neutralization Solution (DMEM, 50% FBS, 1%PSA)

DMEM - Dulbecco's Modification of Eagle's Medium (Corning cellgro®, Manassas, VA)

FBS - Fetal Bovine Serum (Atlanta Biological, Atlanta, GA)

PSA - Penicillin, Streptomycin, Amphotericin B (Corning cellgro®, Manassas, VA)

Cell Culture

hADSC – human adipose-derived stem cell (Invitrogen, Carlsbad, CA)

Complete MesenPRO RS™ Medium: MesenPRO RS™ Basal Medium and MesenPRO RS™ Growth Supplement (Life Technologies™, Carlsbad, CA)

Trypsin EDTA (Corning cellgro®, Manassas, VA)

Cell Scepter (EMD Millipore, Darmstadt, Germany)

Interstitial Cellular Seeding

Seeding Solution (DMEM, 10% FBS, and 1% PSA)

33G needles (Hamilton Company, Reno, NV)

Air pump (Gast Manufacturing, Inc., Benton Harbor, MI)

Conditioning

DMEM, 10% FBS, and 1% PSA

Rotator (developed by Richard Pascal, manufactured by Clemson University Machining and Technical Services)

Bioreactor (developed by Lee Sierad, manufactured by Clemson University Machining and Technical Services)

Histology

4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA)

Hematoxylin & Eosin (H&E) Stain (Thermo Fisher Scientific, Waltham, MA)

DAPI (4'-6-Diamidino-2-phenylindole) Nucleic Acid Stain (Life Technologies™, Carlsbad, CA) and the Vectashield HardSet™ Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA)

Movat's Pentachrome (Poly Scientific R&D Corp., Bay Shore, NY)

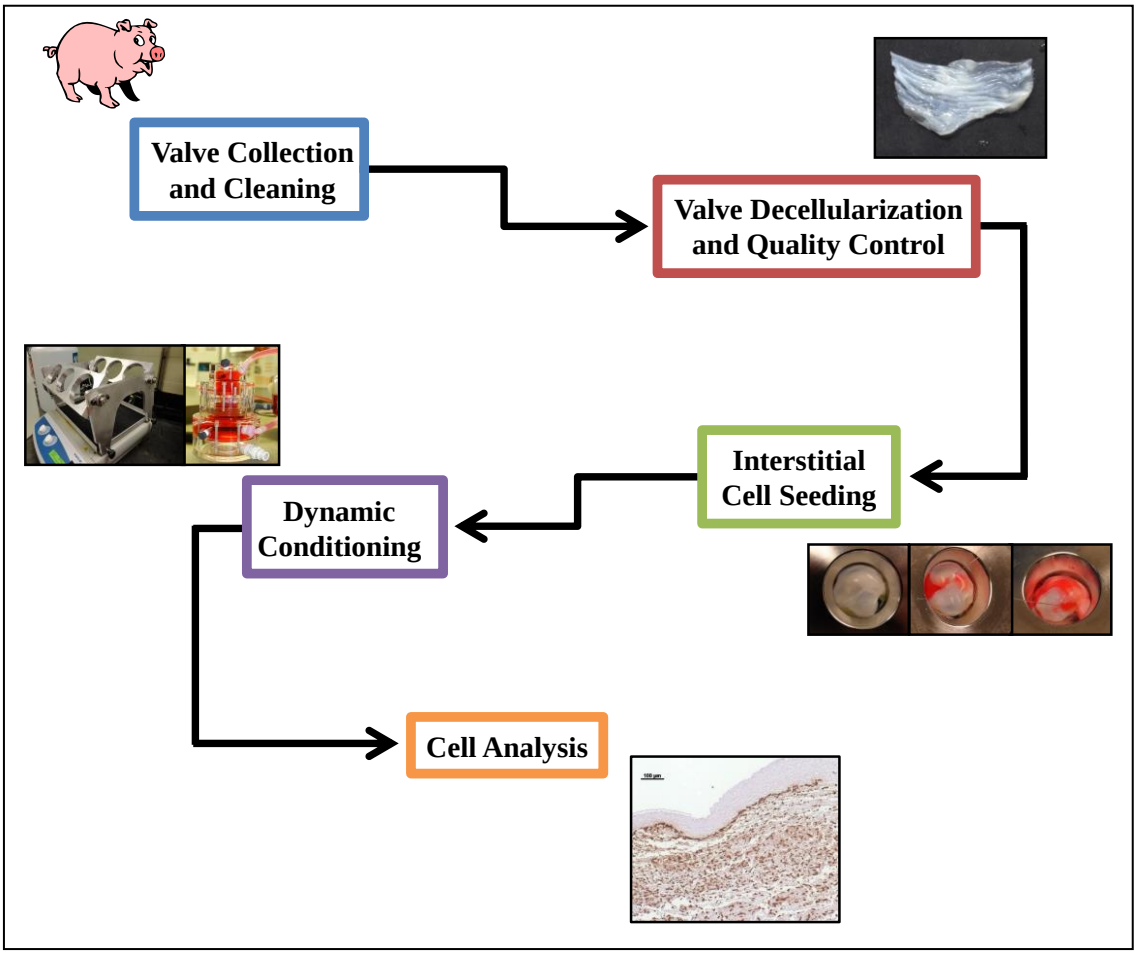
Immunohistochemistry (IHC) antibodies: α-SMA #ab5694, Vimentin #ab92547, prolyl-4-hydroxylase (P4HA3) #ab101657 (Abcam, Cambridge, MA)

Vectastain Elite Kit and ABC diaminobenzidine tetrahydrocholoride peroxidase substrate kit (Vector Laboratories, Burlingame, CA)

Microscope/Camera (Zeiss Axiovert 40CFL microscope using AxioVision Release 4.6.3 digital imaging software, Carl Zeiss MicroImaging, Inc. Thornwood, NY)

3.2 Methods Overview

Figure 7: Methods Overview

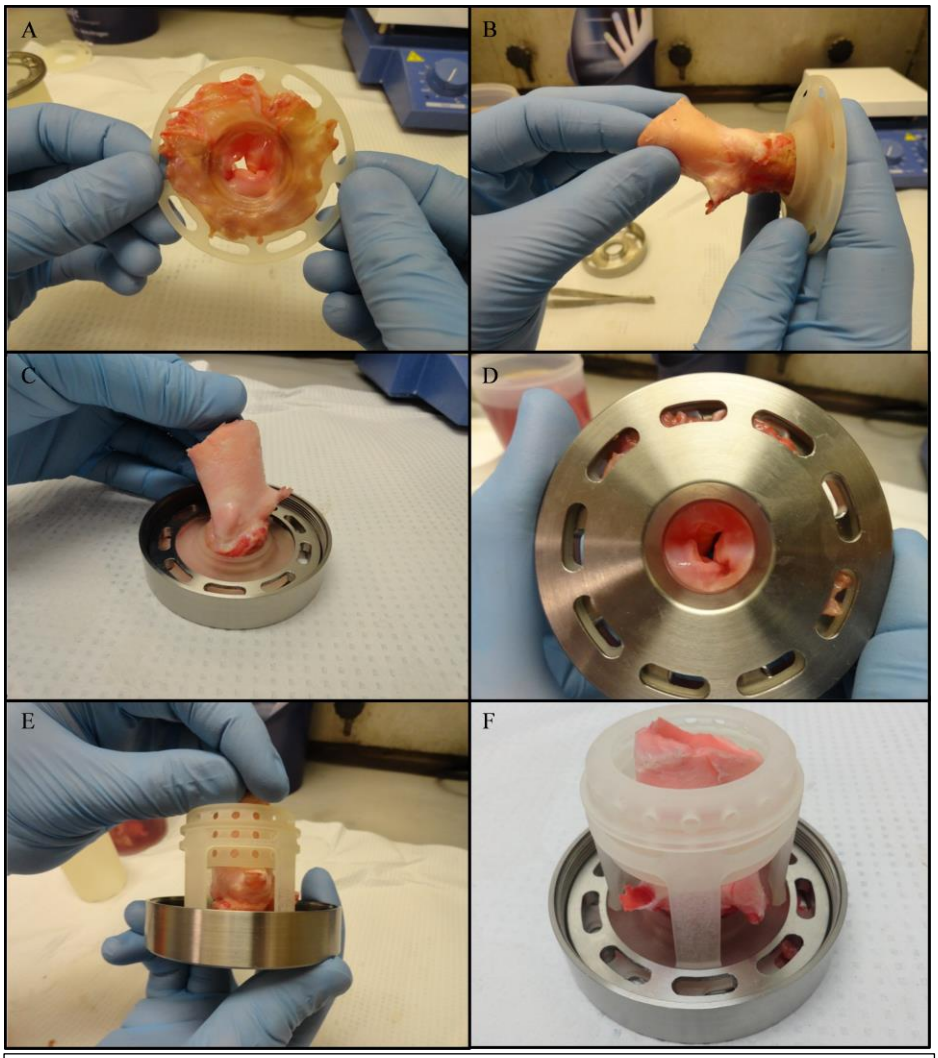


3.3 Experimental Methods

3.3.1 Valve Collection and Cleaning

Aortic valves were harvested from porcine hearts donated by Snow Creek Meat Processing Plant. The aortic valve was dissected out of the heart in such a way as to maintain excess tissue above and below the annulus of the valve. Tissue on the ventricular side of the valve (portions of the mitral valve and surrounding muscle) was needed in order to secure the valve within mounting rings. A large portion of the ascending aorta was also retained for securement in decellularization and conditioning processes. It is important to note that this additional tissue would also serve as a clinical means of suturing the tissue engineered valve into the patient. To prepare the valve for decellularization the loose fascia was removed from around the outside of the aorta and the excess tissue on the ventricular side was thinned to approximately 1.5-3 mm thickness. The coronaries were then isolated from the fat and aorta, and ligated with sutures. After trimming and ligation, the valves were rinsed in ddH_2O and placed into metal mounting rings, as seen in figure 8. These mounting rings are compatible with all devices used for decellularization and conditioning, enabling easy transfer from one system to the other. The valves were stored in ddH_2O at $+4^oC$ in the refrigerator to begin the decellularization process by inducing cell hypotonic shock.

Figure 8: Valve Mounting



A. Excess tissue on the ventricular portion of the valve was spread over plastic inner ring. It was important to center the opening of the valve in the middle of the ring; this would ensure space for the leaflets to open and close properly; B. view from aortic side of the valve; C. plastic ring was secured between the metal base and tightening ring; D. view from the ventricular side showing proper positioning of the valve opening; E. aorta outer legs and aortic inner tube were placed around the aorta extending from the valve; F. final secured mounting structure, the aorta was then sutured to the plastic inner tube to provide stability to the structure.

3.3.2 Valve Decellularization

Decellularization protocol followed that of previously published, Sierad et al. [42]. Briefly, after valves were stored in ddH_2O for at least 22 hours the rest of the decellularization process began with 2 hour incubation in NaOH to relax the extracellular matrix and prime the matrix for cell removal. Followed by three 48 hour washes in decellularization solution, 24 hours in DNase/RNase solution to remove nucleic acids, 2 hours in peracetic acid to sterilize the valves (after this point all steps were done in sterile conditions with sterile filtered solutions and sterile tools), 20 hours in PGG (penta-galloyl glucose) to crosslink the scaffold, and finally stored in PBS. All solution changes during the decellularization were separated by adequate rinsing and incubation in ddH_2O, EtOH, or PBS. During these solution steps, rinses, and incubations the valves remained moving in solution on an orbital shaker plate.

3.3.3 hADSC Culture and Subculture

To thaw and establish hADSCs, cells were removed from cryopreservation in liquid nitrogen, quickly warmed and placed into fresh, warm Complete MesenPRO RS™ Medium. Cells were then plated onto a T-175 flask at a density of about 5000 cells per cm^2. After overnight incubation at 37°C and 5% CO_2 media was replaced. Media change, using Complete MesenPRO RS™ Medium, occurred every 3-4 days to maintain culture and promote proliferation.

To subculture hADSCs, media was aspirated from the flask. The surface was rinsed with PBS, and then aspirated. Trypsin EDTA that was warmed to 37°C was added (at approximately 0.5 mL per 10 cm^2) to detach the cells from the flask. Incubation at 37°C in Trypsin EDTA lasted for approximately 5 minutes or until majority of the cells had detached. When cells were detached Complete MesenPRO Medium was added (approximately 1.5x the volume of Trypsin EDTA used). The cell suspension was transferred to a 50 mL conical tube and centrifuged at 210 x g for

5 minutes to form the stromal vascular fracture (cell pellet). Complete MesenPRO RS™ Medium/Trypsin EDTA mixture was aspirated out of the conical, leaving the pellet intact. Seeding solution was then used to resuspend the cell pellet for counting. The cell scepter was used to count the cells, from which the total number of cells was calculated. Cells were resuspended at the desired density (about 5000 cells per cm^2) and plated. MesenPRO RS™ Medium was replaced every 3-4 days.

3.3.4 Neutralization

After the valves were decellularized and PGG-fixed, they remained in sterile 1x PBS until use. Before the valves could be seeded they underwent neutralization incubation. Valves were incubated in rotating chambers filled with approximately 120mL of neutralization solution for 12-22 hours at 37°C in the incubator. This step was used to neutralize any unbound PGG and enhance the valve scaffold by introducing growth factors prior to seeding.

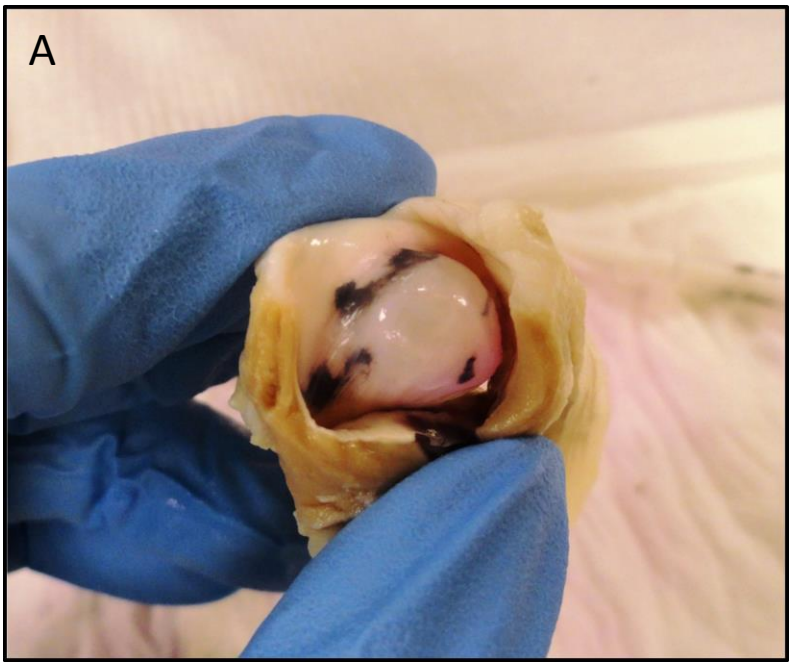
3.3.5 Interstitial Cell Seeding

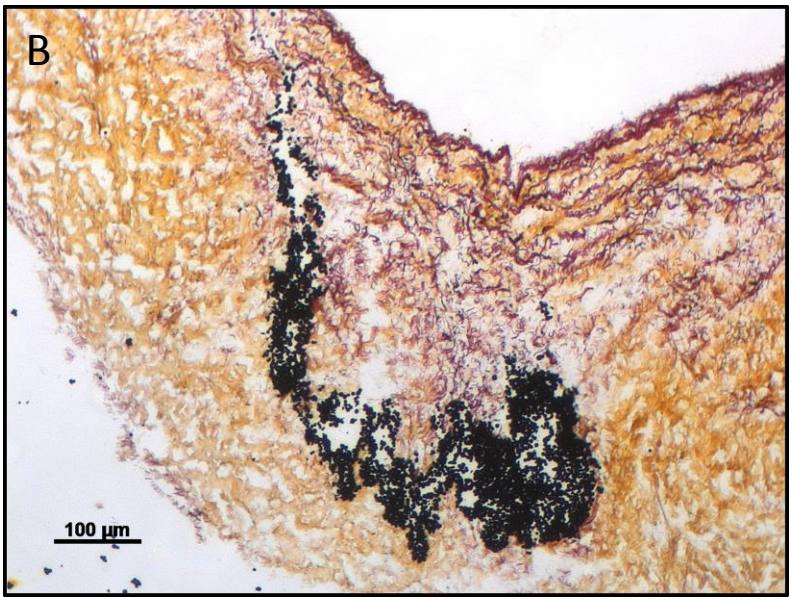
3.3.5.1 Study 1: Injection Testing with Carbon Nanoparticles

Initial injection testing was performed using carbon nanoparticles suspended in DMEM. The carbon first needed to be passivated; dry carbon particles were added to PBS , vortexed several times, bath sonicated for 5 minutes, then centrifuged at 1500 rpm for 5 minutes. A pellet of carbon was formed at the tip of the conical, PBS was removed by vacuum, and pellet was resuspended in FBS by vortexing several times. Particles were left in FBS overnight at room temperature. The following day the solution was centrifuged at 1500 rpm for 5 minutes. Pellet of carbon was formed, FBS removed by vacuum, pellet resuspended in DMEM by vortexing several times. This final injection solution was stored in +4°C refrigerator until use.

Carbon solution was injected using a 30 GA needle either directly into the cusp or indirectly into the cusp through the aortic wall. The injection of carbon was an effective method to visualize the way in which cells would fill the cusp. Figure 9 shows both macro and micro images of the carbon within the scaffold. It was noted that the carbon did not spread in the cusp much farther than the injection site. Alternate methods of injection were proposed to obtain more complete filling of the cusp, but it was really the separation of the cusp layers that would facilitate the spreading of solution throughout the structure.

Figure 9: Carbon Particle Injection



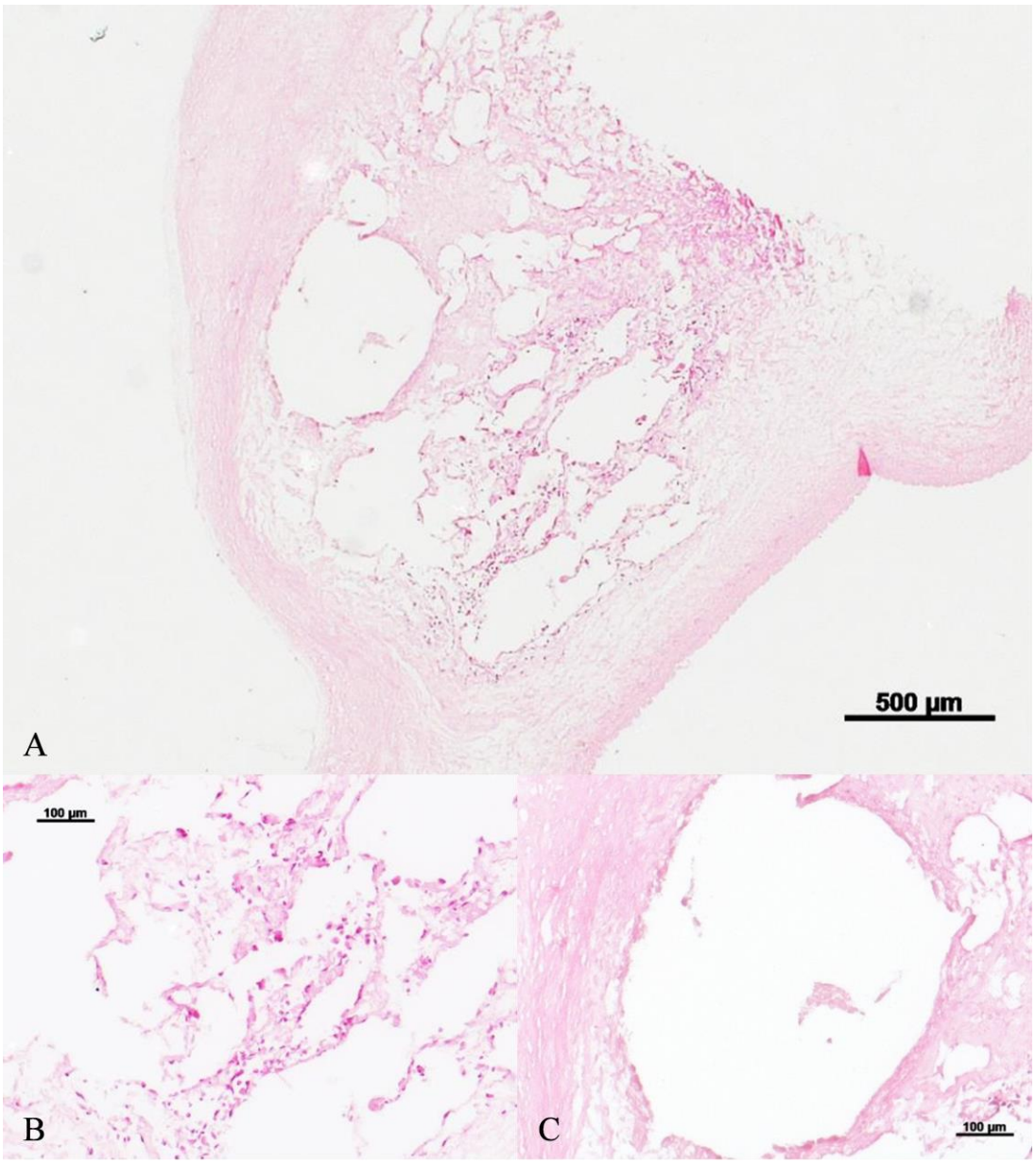


A. Decellularized porcine aortic heart valve injected with carbon nanoparticles (black seen within the cusp structure) both directly and indirectly; B. 10x image of carbon particles (black particulates within cusp structure) within the decellularized cusp structure Movat's stain: nuclei stain bright red, collagen stains yellow, elastin stains deep purple to black

3.3.5.2 Study 2: Air Pre-injection Testing

Pre-injection of air was first attempted using a syringe filled with air, but the pressure generated by hand was not sufficient to achieve desired separation of cusp layers. An air pump was tested and found that a pressure of 15-20 psi would provide adequate expansion of the cusps. The moment that the tip of the needle penetrated the top layer of the cusp the structure expanded and separated the cusp layers creating a void in which cell suspension could then be injected. Figure 10 clearly displays the pockets within the cusp that were created. The base of the cusp was seen to yield the most complete air expansion, and therefore the most complete cell seeding.

Figure 10: Air Pre-Injection

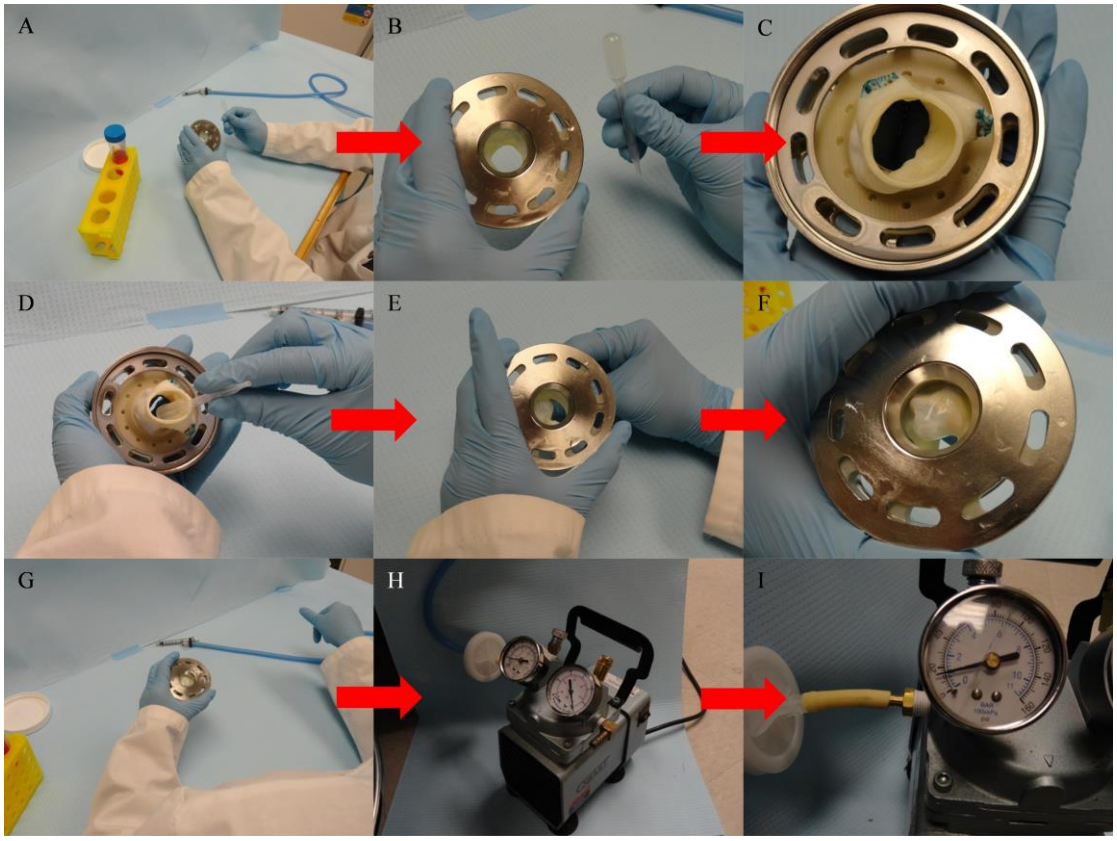


A. 2.5x image of the base of the cusp structure; pre-injection of air into the base of the cusp inflated and created pockets within the scaffold; B. 10x image displaying cells that had been injected after air inflation; C. 10x image displaying size of defect created in the scaffold. H&E stain: nuclei stain purple, ECM components stain pink.

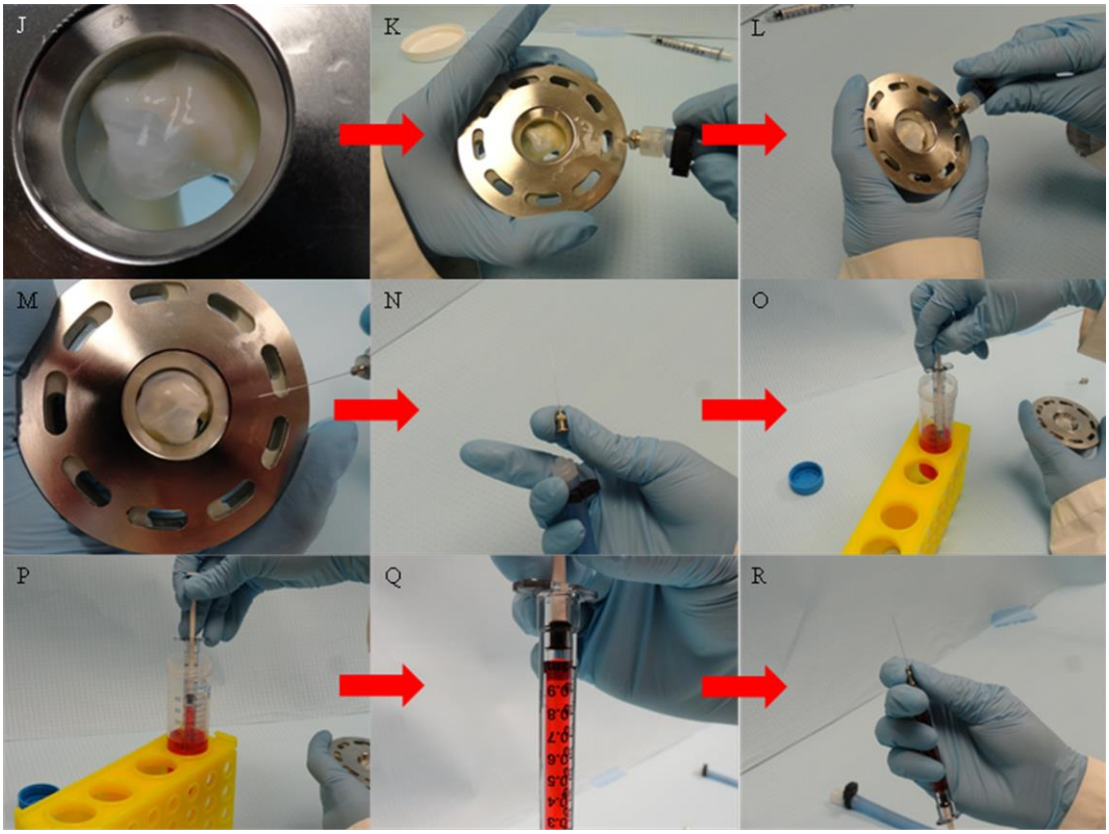
3.3.5.3 Study 3: Injection Protocol

All sterile materials were prepared prior to seeding in either Ethylene Oxide or Autoclave at 121^{o}C. Rotating chambers and bioreactors were assembled before any cell work occurred. Desired amount of cells (about 4 million per cusp) were passaged and stored in 50 mL conical at a concentration of about 8 million cells per mL of seeding solution in the incubator until use. Valves that were designated as those that would undergo bioreactor conditioning were secured in mounting rings and the aorta sutured to the aorta inner tube. The valves that were designated as those that would undergo rotational conditioning were only secured in the mounting rings. Cells were removed from the incubator and mixed by inverting the conical. Syringes were pre-loaded with 1 mL of cell suspension (approximately ½ mL per cusp, 4 million cells). The pictures in figure 11 illustrate the following injection processes. Air pump and 33 GA x 1 ¼ inch needle were used to inflate the base and free edge of the cusp with approximately 1-2mL of sterile air. A 33 GA x 1 ¼ inch needle was then attached to the pre-loaded syringe and cell suspension was injected into inflated portions of the cusp (about 0.25 mL per injection site). This same injection procedure was repeated for each cusp in each valve. Specified valves were either transferred into the bioreactor or rotator for conditioning. The bioreactor was filled with about 700 mL of media and the rotating chamber filled with about 120 mL of media. Bioreactor pressures and rotator speeds were monitored daily and media was changed every 3-5 days as needed.

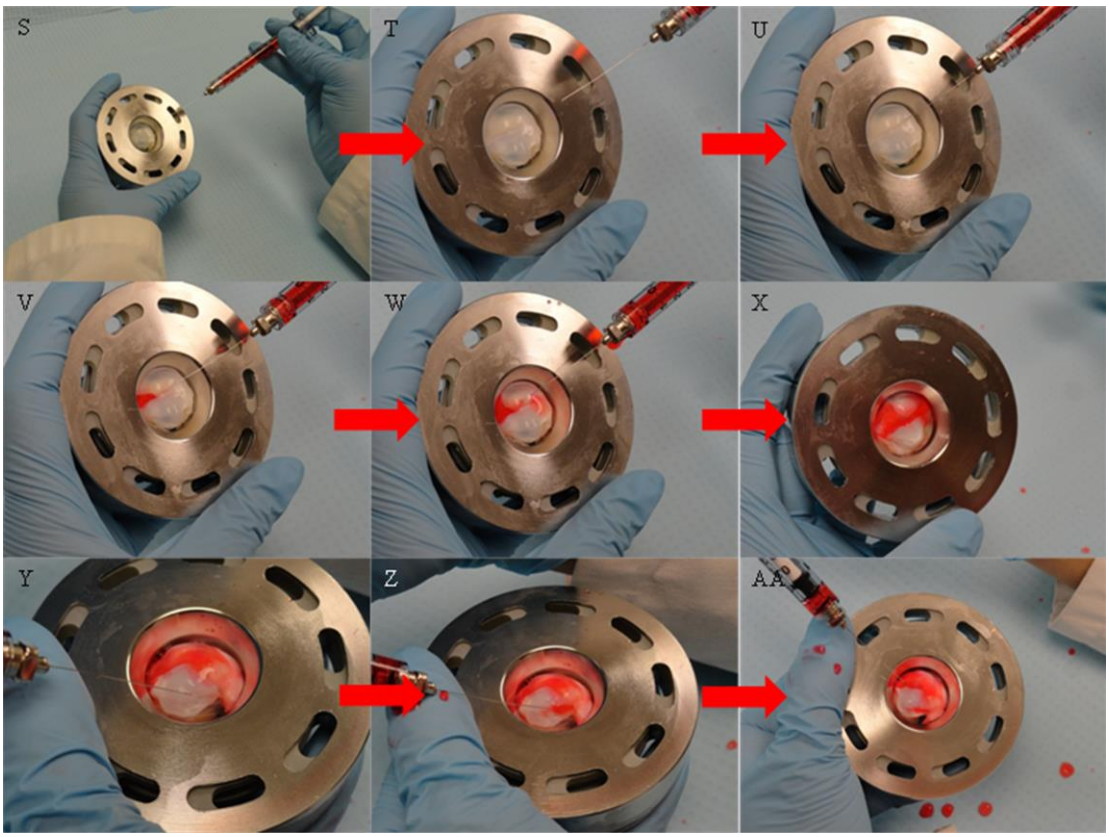
Figure 11: Injection Procedure Pictorial



A. Collected all materials for air injection; **B**. small bulb pipette and valve that was secured in metal mounting rings; **C**. view when looking down the aorta into the valve; **D**. the bulb pipette was inserted from this side of the aorta in such a manner that it filled the cusp; **E**. once the pipette tip was inside of the cusp, the valve was reversed so that the observer was looking at the valve from the ventricular side; **F**. the cusp was then stabilized and stretched outward, gaining better access for injection; **G**. valve and pipette were all be held within one hand, with the other kept free to perform the injection; **H**. pump was attached to a sterile filter and tubing; **I**. pressure was maintained between 15 and 20psi



J. Close up of stretched cusp; **K**. needle attached to the tubing was held in opposite hand; **L.** needle tip was carefully advanced into cusp at the base (where the cusp meets the aortic wall; **M**. when cusp structure began to inflate the needle was kept in until desired inflation was achieved and then removed; **N**. needle was then detached from the tubing by its luer connection; **O**. with the valve still in one hand, the 1mL syringe was filled with cell suspension; **P**. filling of the syringe was done with one hand, the plunger pulled upwards between thumb and forefinger; **Q**. approximately 0.5mL was used per cusp (4 million cells), one syringe was sufficient for two cusps; **R**. needle was then reattached to the syringe luer



S. Gripped syringe with one finger on the end of the plunger and the others secured around the body; **T.** cell suspension was administered at cusp inflation site; **U**. needle tip was carefully advanced into cusp; **V.** once the needle was in place the plunger was depressed, administering cell suspension into the cusp; **W**. continued to administer suspension until the solution filled the entire inflated base; **X**. approximately 0.25-0.5mL of cell suspension per injection site was used; **Y**. needle tip was then carefully inserted into the tip portion of the cusp; **Z**. depressed plunger to administer approximately 0.25mL of cell suspension and removed needle; **AA**. Cusp was then considered seeded and the process repeated for each of the other cusps

3.3.6 Dynamic Conditioning

Bioreactor

For the first 24 hours that the valves were in the bioreactor pressure was kept at a minimum; just enough to have slight opening and closing of the leaflets. Over the following 14 days pressure was steadily increased to simulate pulmonic conditions (25-10 mmHg) and 65 beats per minute. Valves were kept at these conditions for 10 more days and the study concluded after 24 total days within the bioreactor. Media was changed every 3-4 days.

Rotator

Immediately after the valve chambers were secured in the rotator the shaker plate to which it was attached was started at a level of 2.5 and the rotator was set to speed of 5 rpm. When looking at the valves while rotating, movement of the cusps was observed. The speed of rotation was increased over 10 days until 10 rpm was reached and the shaker plate was at level 4. The remaining 14 days of the study the valves remained at these conditions, and on day 24 the study was concluded. Media was changed every 3-4 days.

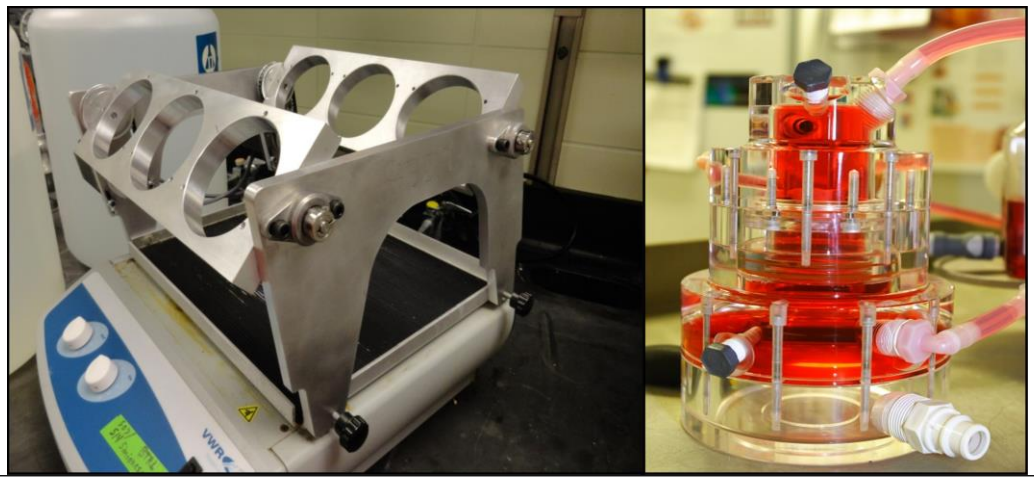
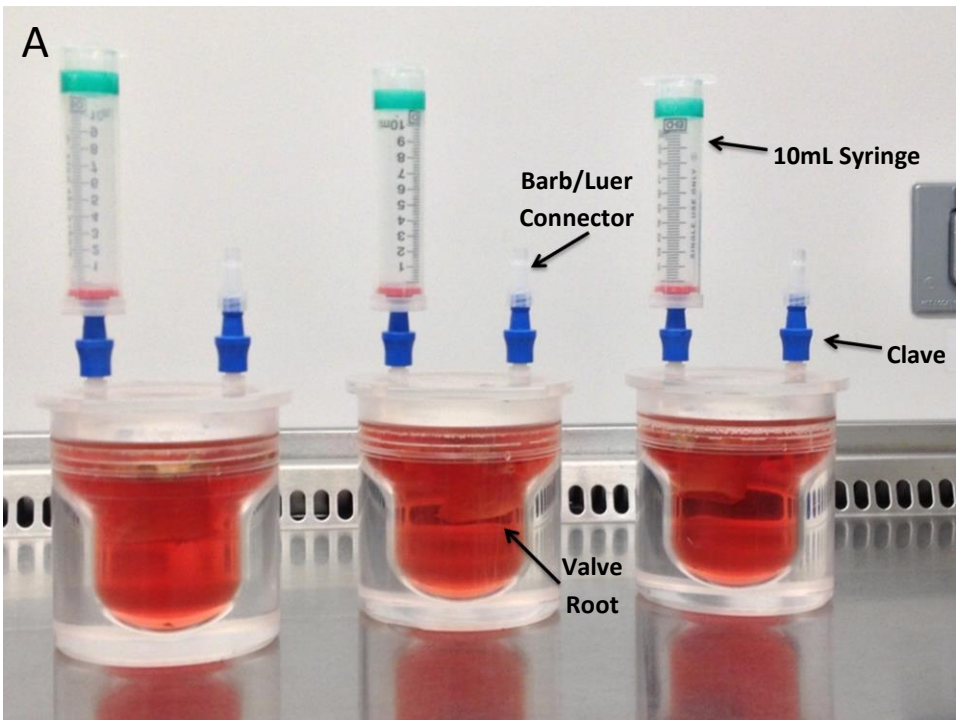


Figure 12: Rotator and Bioreactor Conditioning Systems - Rotating chamber (developed by Richard Pascal) attached to shaker plate (left) [43]. Clemson Heart Valve Bioreactor v1 (developed by Lee Sierad) (right) [42].

Figure 13: Media Replacement in Rotating Chambers



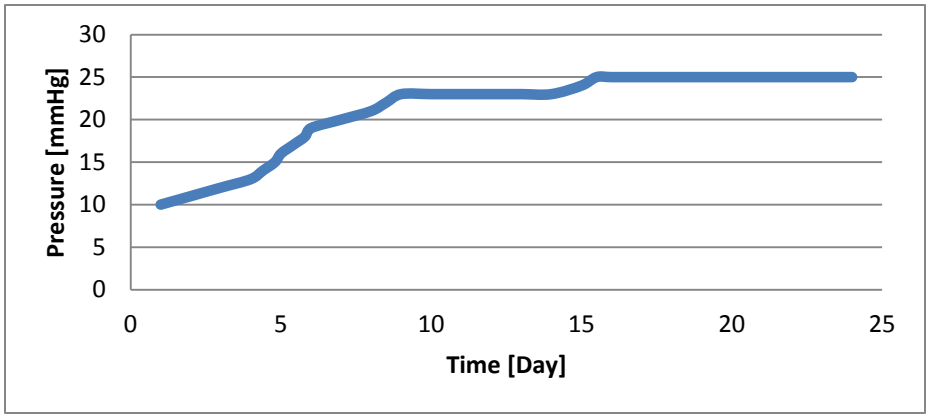
A. Valves within seeding chambers; media change was essential for studies beyond a 3-4 day time period; in order to remove the media, barb/luer ended connectors were placed on claves that were secured to the lid of the chamber, this barb was then connected to a vacuum pump which pulled the media out; to replace media a 10mL syringe with luer connection was placed onto the second clave that was secured to the chamber lid, then media was added; B. Removal of media through vacuum suction.

Table 1: Bioreactor (Left) and Rotator (Right) Conditioning Regimes

Time [day]	Pressure [mmHg]
1	10
2	11
3	12
4	13-15
5	16-18
6	19
7	20
8	21-22
9	23
10	23
11	23
12	23
13	23
14	23
15	24-25
16	25
17	25
18	25
19	25
20	25
21	25
22	25
23	25
24	25

Time [day]	Speed [rpm]	Shaker
1	5	2.5
2	5	3
3	5	3
4	6	3
5	6	3
6	6	3
7	7	3
8	8	3.5
9	8	3.5
10	9	3.5
11	10	4
12	10	4
13	10	4
14	10	4
15	10	4
16	10	4
17	10	4
18	10	4
19	10	4
20	10	4
21	10	4
22	10	4
23	10	4
24	10	4

Figure 14: Bioreactor Pressure Regime



3.3.7 Histological Analysis

Cusp tissues were dissected out of the valves and placed in 4% PFA. Processing of tissues was then done using a Tissue Tek® Processor, followed by embedding, and sectioning (at 4um). Orientation of the cusp sections were in the radial direction. All histological analyses were carried out according to the product specifications. Brief descriptions of protocols and solutions have been listed below.

Hematoxylin & Eosin Staining Procedure

Solutions:

1. Hematoxylin I
2. Clarifier I
3. Bluing Reagent
4. Eosin-Y

Staining Procedure:

1.	Xylenes	10 dips
2.	Xylenes	5 min
3.	100% EtOH	10 dips
4.	100% EtOH	1 min
5.	95% EtOH	10 dips
6.	95% EtOH	1 min
7.	Tap Water	Till "sheeting" action occurs
8.	Distilled Water	1 min
9.	Hematoxylin	7 min
10.	Tap Water	Till clear
11.	Clarifier	5 dips
12.	Tap Water	Till "sheeting" action occurs
13.	Bluing Reagent	1 min
14.	Tap Water	1 min
15.	95% EtOH	10 dips
16.	Eosin	35 sec
17.	95% EtOH	10 dips
18.	95% EtOH	10 dips
19.	100% EtOH	10 dips
20.	100% EtOH	1 min
21.	100% EtOH	3 min
22.	Xylenes	10 dips
23.	Xylenes	5 min

DAPI Staining Procedure

Sections were deparaffinized and hydrated to distilled water:

1.	Xylenes	10 dips
2.	Xylenes	5 min
3.	100% EtOH	10 dips
4.	100% EtOH	1 min
5.	95% EtOH	10 dips
6.	95% EtOH	1 min
7.	Tap Water	Till "sheeting" action occurs
8.	Distilled Water	1 min

Vectashield DAPI Hard Set ™ (1.5ug/mL) applied directly to sections and coverslipped.

Movat's Pentachrome Staining Procedure

Solutions:

1. Weigert's Iron Hematoxylin Sols A&B
2. Alcian Blue 1% in 1% Acetic Acid
3. Resorcin – Fuchsin Working Solution
4. Woodstain Scarlet – Acid Fuchsin
5. Saffron Du Gratinais 3% Alcoholic
6. Alkaline Alcohol
7. Acetic Acid 0.5% Aqueous
8. Phosphotungstic Acid 5% Aqueous

Staining Procedure:

1. Deparaffinize and hydrate to water
2. Rinse thoroughly in distilled water
3. Place in Alcian Blue 1% in 1% Acetic Acid for 20 minutes
4. Wash in running water for 3 minutes
5. Place in Alkaline Alcohol (pH must be over 8) for 2 hours
6. Wash in running water for 10 minutes
7. Rinse in 70% Alcohol
8. Place in Resorcin-Fuchsin Working Solution for 16 hours
9. Wash in running water for 10 minutes
10. Rinse in distilled water
11. Place in Weigert's Hematoxylin Working Solution for 15 minutes
12. Rinse in running water
13. Rinse in distilled water
14. Place in Woodstain Scarlet – Acid Fuchsin Solution for 5 minutes
15. Rinse in Acetic Acid 0.5% Aqueous
16. Differentiate in Phosphotungstic Acid 5% Aqueous for 10-20 minutes, until the collagen is pale pink and the background substance, which is covered by the red, is bluish again.
17. Rinse in Acetic Acid 0.5% Aqueous
18. Rinse thoroughly in three changes of Absolute Alcohol
19. Place in Saffron Du Gratinais 3% Alcoholic for 5-15 minutes.
20. Dehydrate in three changes of Absolute Alcohol, and clear in several changes of Xylene. If the collagen is not sufficiently yellow, repeat the staining with Saffron Solution
21. Mount with Permount or any other acceptable mounting medium.

Immunohistochemistry Staining Procedure

Solutions:

1. Tris Buffer Saline (TBS) → 6.05 g TRIS, + 8.95 g NaCl + 1L water, pH to 7.5
2. 0.3% H_2O_2 in 0.3% normal serum in TBS → 1.8 mL TBS + 6ul normal serum + 200ul 3% H_2O_2
3. 0.025% Triton → 25ul Triton + 100mL TBS
4. Normal blocking serum (1.5%) (Vectastatin kit) → 30uL horse normal serum + 2mL TBS
5. TNB buffer → 1mL blocking reagent solution from Western Blot kit) + 19mL TBS
6. Secondary antibody → 30uL normal horse blocking serum + 2mL TBS + 10uL biotinylated IgG
7. ABC complex → provided as Ready To Use from Vector
8. DAB substrate solution → 5mL water + 2 drops Buffer Stock Solution, mix well, then add 4 drops DAB stock solution, mix well, then add 2 drops H_2O_2, mix well
9. Primary antibody → Prepare according to manufacturer's recommendation

Staining

1. Deparaffinize and hydrate sections to water
2. Place slides in 10mM citric acid monohydrate (pH 6.0)
3. Place in microwave and put power setting to 5. Microwave for 5 minutes, check temperature at 2.5 minutes. Repeat for a total of 20 minutes, the last 10 minutes of microwaving will yield a citric acid solution temperature between 90 - 100°C (monitor for evaporation)
4. Allow buffer and slides to cool to room temperature (~30-60 minutes)
5. Rinse slides twice in TBS for 5 minutes each.
6. Circle sections with a Vector "immedge pen" – make sure sections don't dry
7. Rinse twice in 0.025% Triton for 5 minutes to permeabilize tissue
8. Rinse once in TBS for 5 min
9. Incubate sections in normal blocking serum for 45 minutes at room temperature to block non-specific binding sites
10. DO NOT RINSE SLIDES. WICK THEM WELL (blott excess serum from sections)
11. Apply primary antibody made in TNB buffer. USE TBS FOR NEGATIVE CONTROL. Incubate at room temperature for 1 hour or overnight at 4°C in humidified chamber
12. Following incubation, rinse twice in TBS for 5 minutes each
13. Block (quench) the endogenous peroxidase with 0.3% H_2O_2 in 0.3% normal serum in TBS for 30 minutes
14. Rinse twice in TBS for 5 minutes each
15. Apply secondary biotinylated antibody for 30 minutes at room temperature
16. Rinse twice in TBS for 5 minutes
17. Apply R.T.U. ABC complex for 30 minutes at room temperature (Avidin – horseradish peroxidase complex)
18. Rinse twice in TBS for 5 minutes
19. Develop with DAB for 2 minutes (or determine the time under the microscope by applying DAB to one section at a time and allowing it to develop at 1 minute intervals – stop reaction by dipping repeatedly in water
20. Rinse in tap water for 5 minutes
21. Counterstain in diluted hematoxylin (50% hematoxylin, 50% water) for 1 minute
22. Rinse in water
23. Dehydrate, clear, and mount

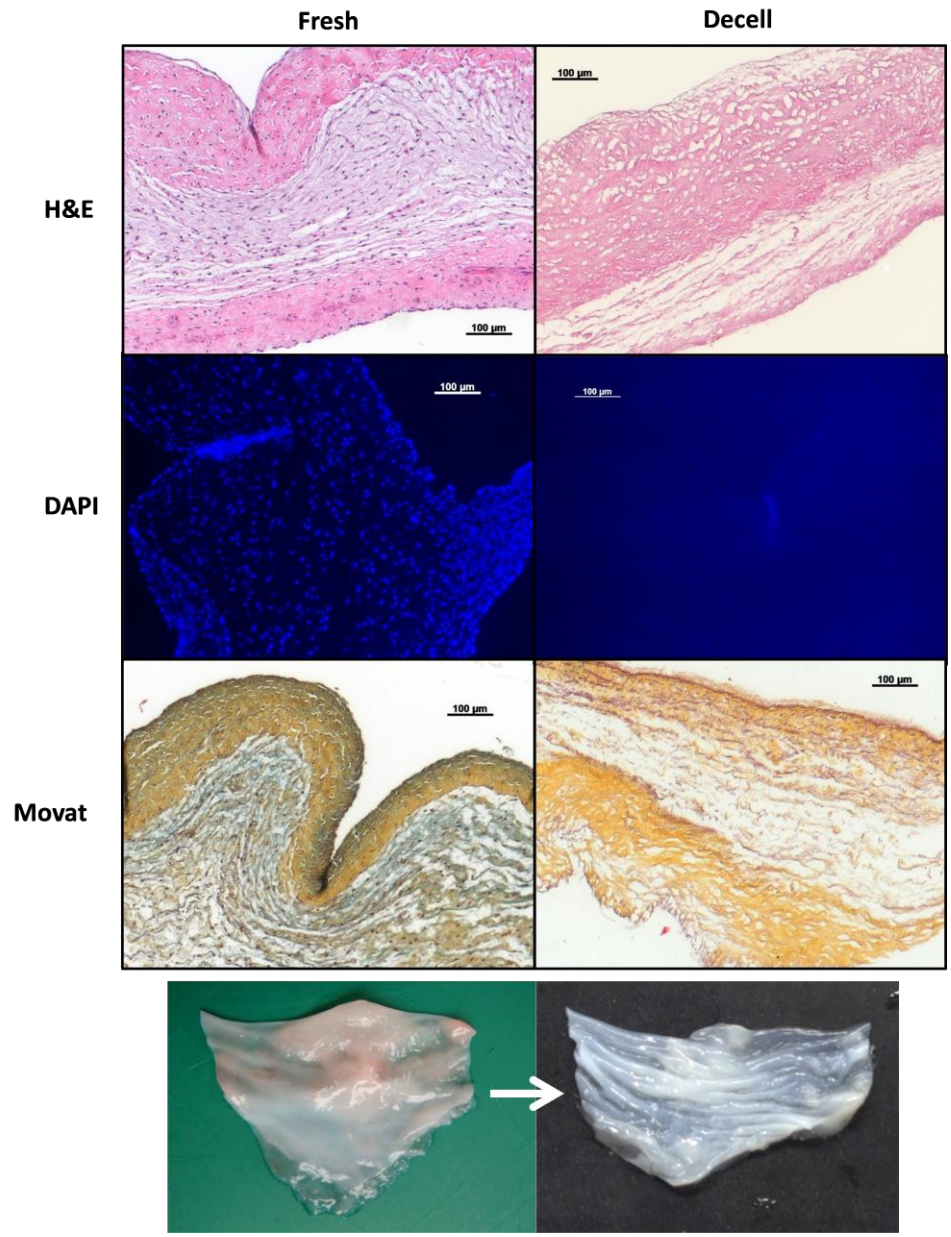
3.3.8 Ovine Model In Vivo Study

Partnering with researchers and clinicians in Romania, large animal implantation of interstitially seeded aortic heart valve constructs studies have begun. Lipoaspirate is collected from the fatty tissue around the neck and shoulder blades of the sheep. The adipose-derived stems cells can then be isolated and cultured. Valves are decellularized by perfusion method, fixed with PGG, and neutralized in DMEM, FBS, PSA solution. The scaffolds are then seeded interstitially by the injection method that is displayed in figure 11. The method was taught to several of the people who are directly working on the project in Romania, two researchers and one clinician. These individuals learned the cell injection technique quickly and have been able to reproduce the process on their own. Thus far three sheep have been implanted with injection seeded aortic heart valves within a pulmonary conduit.

Figure 15: Ovine In Vivo Study – One of the sheep that was implanted with an interstitially seeded aortic heart valve. Visible incision and shaved areas from where the adipose tissue was retrieved are seen.

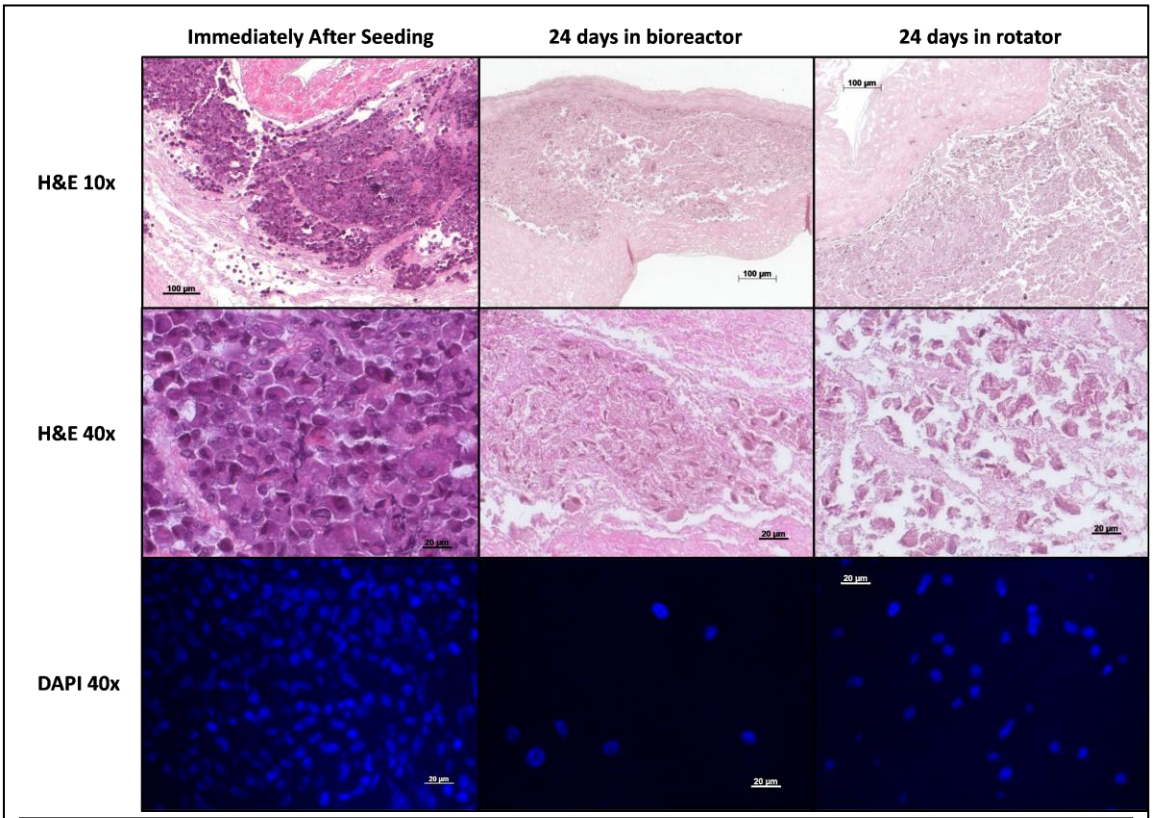
CHAPTER 4: RESULTS

Figure 16: Decellularization Analysis



Comparison of fresh cusp tissue (left) and decellularized cusp tissue (right). H&E (top): nuclei stain purple, ECM components stain pink. DAPI (2nd from top): nuclei stain bright blue. Movat's Pentachrome (3rd from top): nuclei stain red, collagen stains yellow, elastin stains deep purple to black, GAGs stain blue. Gross images of cusps after being dissected out of the aortic valve structure (bottom).

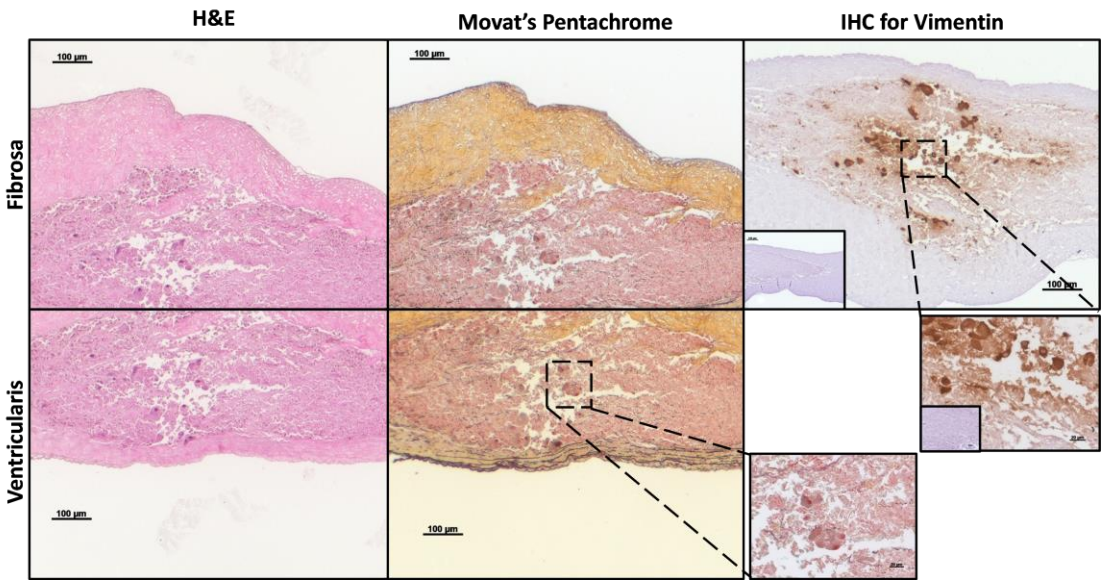
Figure 17: Cusp Immediately After Seeding, 24 Days in Bioreactor, and 24 Days in Rotator: H&E and DAPI Staining



Comparison of seeded scaffolds over various time points; immediately after seeding (left), 24 days in bioreactor (middle), and 24 days in rotator (right). H&E (top 2 rows): nuclei stain purple, ECM components stain pink. DAPI (bottom): nuclei stain bright blue.

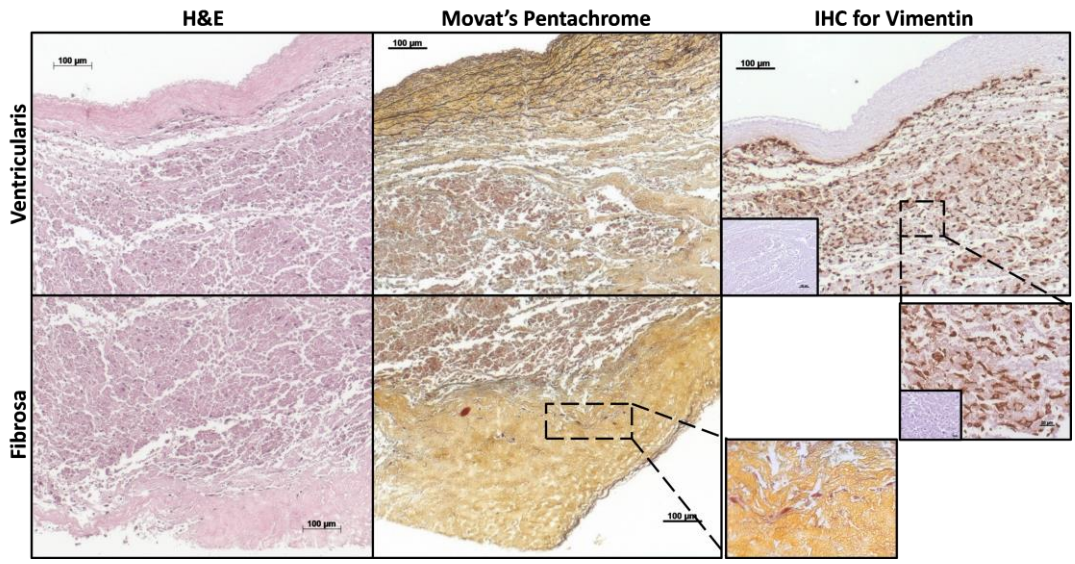
Images of the cusp immediately after seeding confirm that a large bolus of cells was introduced into the structure. From the H&E images of the cusp after 24 days in the bioreactor, there appear to be many cells retained within the cusp, but when comparing the DAPI image of that same location only a few cell nuclei are visible. Both the H&E and DAPI images of the cusp after 24 days in the rotator the cell population is characteristic of native (fresh) aortic heart valves.

Figure 18: Cusp after 24 Days in the Bioreactor: H&E, Movat's Pentachrome, and IHC (Vimentin) Staining



Images depicting the structure of the fibrosa (top) and ventricularis (bottom) after 24 days of bioreactor conditioning. H&E (left): nuclei stain purple, ECM components stain pink, Movat's Pentachrome (middle): nuclei stain bright red, collagen stains yellow, elastin stains deep purple to black, fibroid/muscle stains red. IHC for Vimentin (right): brown staining is positive expression counterstain with 50/50 hematoxylin.

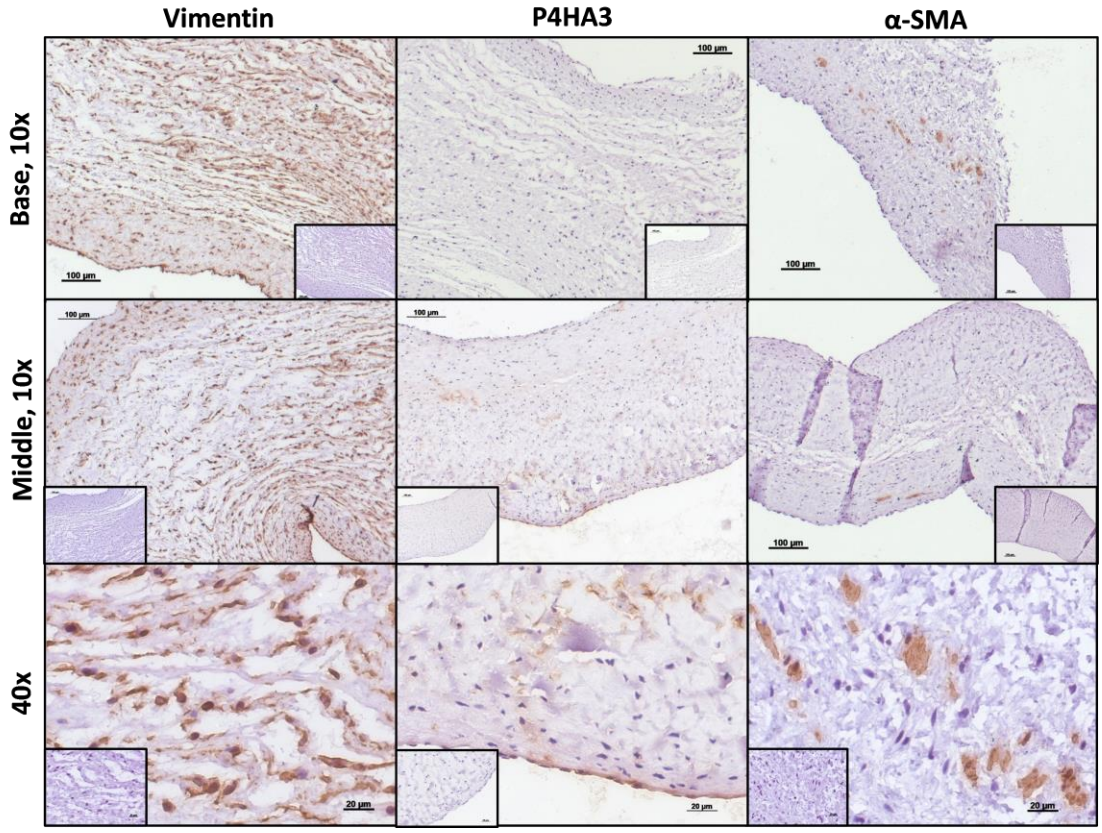
Figure 19: Cusp after 24 Days in the Rotator: H&E, Movat's Pentachrome, and IHC (Vimentin) Staining



Images depicting the structure of the ventricularis (top) and fibrosa (bottom) after 24 days of rotator conditioning. H&E (left): nuclei stain purple, ECM components stain pink; Movat's Pentachrome (middle): nuclei stain bright red, collagen stains yellow, elastin stains deep purple to black, fibroid/muscle stains red; IHC for Vimentin (right): brown staining is positive expression, counterstain with 50/50 hematoxylin.

It was observed that the cells created some sort of matrix over the 24 day time period in vitro. The higher magnification (40x) panel of the Movat's Pentachrome stain displays the migration of cells into the fibrosa

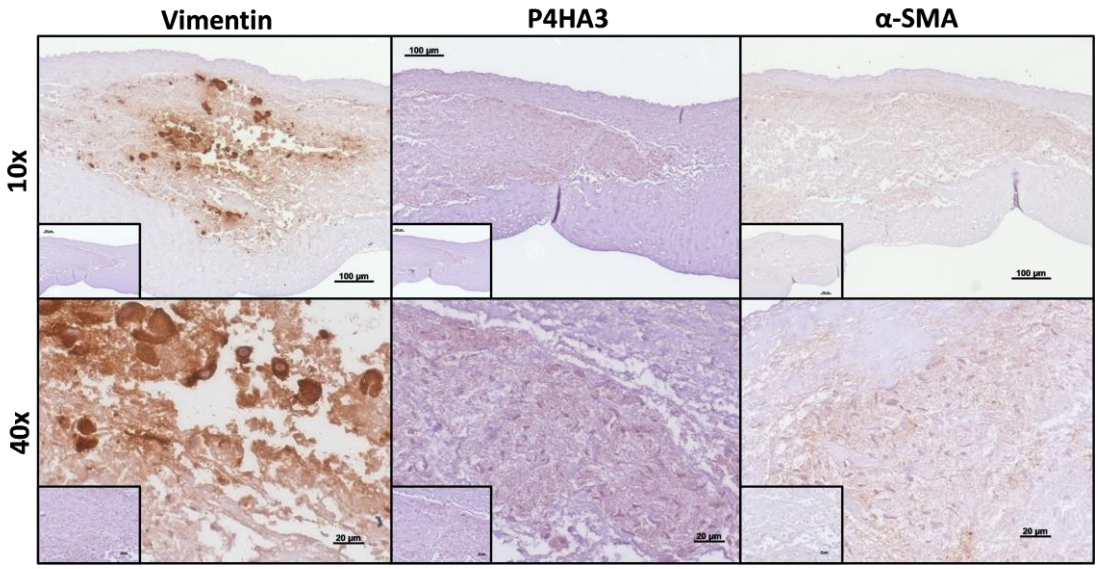
Figure 20: Fresh Cusp: IHC for Vimentin, Prolyl-4-hydroxylase, and α-SMA



Immunohistochemistry (IHC) of fresh aortic heart valve cusps; brown staining indicates positive expression of the marker of interest. Vimentin (left), Prolyl-4-hydroxylase (middle), α-SMA (right).

The cells within the native (fresh) aortic heart valve cusp positively stain for Vimentin throughout the entirety of the structure, denoting a fibroblast-like phenotype. Prolyl-4-hydroxylase (P4HA3) would be indicative of any new collagen formation in the cusp structure; native cusps express little to no P4HA3. As discussed in section 2.4, α-smooth muscle actin (α-SMA) is expressed by those VICs that are considered 'activated'; native cusps were shown to express little to no α-SMA.

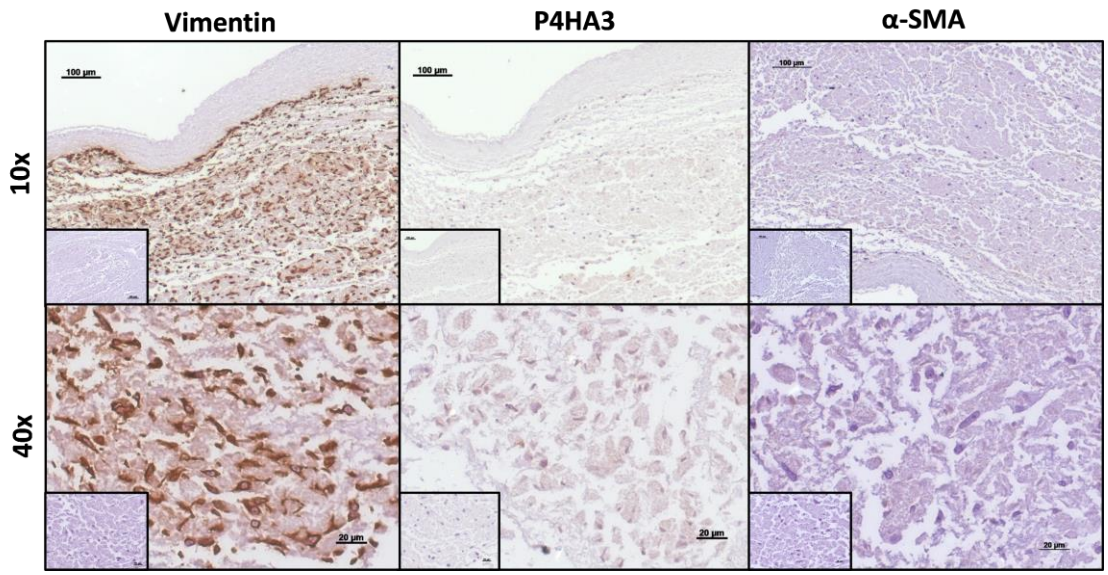
Figure 21: Cusp after 24 Days in the Bioreactor: IHC for Vimentin, Prolyl-4-hydroxylase, and α-SMA



Immunohistochemistry (IHC) of cusps after bioreactor conditioning for 24 days; brown staining indicates positive expression of the marker of interest. Vimentin (left), Prolyl-4-hydroxylase (middle), α-SMA (right).

Staining of Vimentin had an over development of the peroxidase step during staining protocol in the mid-region of the section, but the individual cells staining positive for Vimentin can still be distinguished due to direct comparison of DAPI images and IHC images; this comparison can be made because serial section were taken. No expression of either P4HA3 or α-SMA was observed.

Figure 22: Cusp after 24 Days in the Rotator: IHC for Vimentin, Prolyl-4-hydroxylase, and α-SMA



IHC of cusps after rotational conditioning for 24 days; brown staining indicates positive expression of the marker of interest. Vimentin (left), Prolyl-4-hydroxylase (middle), α-SMA (right).

Positive expression of Vimentin is completely characteristic of that seen in native aortic heart valve cusps. No expression of either P4HA3 or α-SMA was observed.

CHAPTER 5: DISCUSSION

5.1 Decellularization

Histological evaluation, figure 16, showed excellent decellularization of the cusp tissue, which was comparable to the results seen in the publication of reference for the decellularization procedure [42]. However, this protocol is still insufficient in the complete decellularization of the aortic wall, but much promise has been observed in a perfusion decellularization technique. Complete decellularization is essential when looking into the clinical use of these constructs. Catastrophic failure of the Synergraft^{TM} valve construct was observed in the clinical study discussed in Section 1.5, in which the patient's extreme immune response was triggered by inadequate decellularization of the xenogeneic scaffold [18].

Analysis on if the more robust perfusion decellularization process damages the cusp tissue will need to be performed. The ECM structure post-decellularization must still be strong enough to withstand cell seeding methods and physiological pressures. A decrease in the mechanical properties of the cusps could hinder the proper seeding of cells via the suggested injection technique, and could eventually have an impact on cell retention within the structure.

5.2 Cell Sourcing

When determining the cell source to be used in tissue engineering research it is important to consider the clinical accessibility of obtaining those cells. Sources can be broadly grouped into two types, those deemed unreasonable and those that are reasonable cell sources. Examples that would be considered unreasonable include: embryonic, neonatal, and valvular interstitial cells. Some of these sources are cause for ethical concern, but many are simply unable to be harvested because they are cell types that either are no longer found in adults or cannot not be retrieved

from the their location in-situ. For example, heart valves are deemed non-sacrificial tissues due to the importance of their function, therefore autologous VICs cannot be collected for recellularization purposes [32]. Reasonable cell sources include those that are readily available and those that can be derived from readily available sources like induced pluripotent stem cells. Any autologous adult cell source would be considered, including: bone marrow-derived stem cells, endothelial cells, fibroblasts, and adipose-derived stem cells. Although bone marrow-derived and induced-pluripotent are considered reasonable, sources like these and others similar can be either very painful for the patient or potentially harmful due to methods used to induce their stemness.

In order to satisfy the inadequacy of using clinically irrelevant cell types, the use of easily acquired and readily available ADSCs is very promising due to their recorded ability to differentiate into a plethora of cell lineages. Specifically they have been shown to differentiate into fibroblast-like cells; expressing markers characteristic of valvular interstitial cells [29]. As seen in figure 5, ADSCs are isolated from lipoaspirate that is obtained through route liposuction procedures. Also, an abundant number of these cells can be found in relatively small amounts of fatty tissue, reducing the amount of total tissue that needs to be collected.

5.3 Interstitial Cell Seeding Method

Several research groups have reported successful recellularization of the cusp structure within a valve scaffold, but it is important to review the ways in which these groups have fallen short of a clinically relevant replacement. Various processes require extensive scaffold preparation, seeding, and conditioning protocols. When relying upon seeding approaches by cell infiltration it is the long conditioning steps that are needed to observe any cell migration. It has even been noted that after weeks of in vitro conditioning cell migration was not accomplished. Looking at groups that

have published in vivo repopulation of the interstices of the cusp, they have not completely characterized the cell type that has repopulated the structure. Many do not report on results of inflammatory or immune response; it is important to know if the cells that are seen in the structure are macrophages or other immune cells. Another hallmark of most in vivo data thus far is the extensive expression of α-SMA throughout the cusp structure. Even after several months in vivo cell expression of α-SMA persists [27], the indication of a myofibroblastic phenotype (activated-VIC). The same is seen in the literature on in vitro conditioning resulting in α-SMA expression [24, 25]. As discussed in Section 2.4, the persistent expression of α-SMA is correlated with the development of valvular pathologies.

These along with many other reasons is why there is a need to develop a technique that is fast, simple, and effective in order to obtain a clinically relevant heart valve replacement. Previous attempts by those in this lab group to recellularize the acellular aortic heart cusp in vitro include such techniques as, applying a charge across the tissue, chemotaxis, and infiltration of cells into the interstices during static and dynamic culture. In testing, each of these techniques was unable to achieve cell infiltration through the cusp tissue. This led to the investigation of cell injection directly into the cusp structure. As this technique was attempted there was a need to create more space between the cusp layers in order to inject a larger bolus of cells. Pre-injection of air into the base of the cusp was necessary in order to achieve this separation of the cusp layers. It was important to note that although this process did create voids within the cusp, the cells that were injected were able to either partially or totally replace the area after conditioning in both the bioreactor and rotator.

As the injection technique was optimized it was found, through both visual observation and histological analysis that the best site for inflation and seeding of the cusp was through the

ventricularis. Reviewing the anatomy of the cusp structure, Section 1.2, the ventricularis is a very elastin-rich region. It was found that this region was the most functionally appropriate area to introduce the 33 GA needle; the elastin fibers were able to provide a 'self-sealing' capability when the needle was retracted. The radially aligned elastin fibers were able to stretch around the needle as it was inserted and then relax back after its removal, in essence, recoil to close the opening created in the cusp. In comparison, when the needle was inserted from the collagen-rich fibrosa region of the cusp, air and cell suspension were able to escape due to the opening that the needle made in the structure. It is not only the fact that the collagen bundles are aligned circumferentially, but most importantly the collagen structure does not lend itself to having this contractile, 'self-sealing' capability.

5.4 Dynamic Conditioning

During the process of engineering a tissue replacement there exists an overarching concept of construct conditioning, whereby the scaffold and cellular components are subjected to physiologically relevant conditions (i.e. temperature, pressure, nutrients, etc.) for some prescribed time length. Those systems constructed to condition tissues are referred to as bioreactors, as discussed in Section 1.5. The conditioning of tissue constructs as a prerequisite for implantation is the direction that the field has heavily emphasized in order to create truly functional tissue replacements [13, 16, 17, 19, 21, 22, 24, 25, 29-30, 32, 40]. Specifically for heart valve tissue engineering, most bioreactor systems attempt to mimic the pulsatile flow of the beating heart. The current work compared the conditioning of a pulsatile-flow bioreactor and a rotating system seen in figure 12.

After valves were conditioned in either bioreactor or rotator systems for 24 days the recellularized cusp sections resembled the structure of fresh (native) aortic heart valve cusps, and they also had

very similar protein and enzyme marker expression/non-expression compared to fresh samples. Overall the highest cell retention and what is believed to be cell proliferation was seen in the cusps that were subjected to the rotational regime. As seen in figure 17, DAPI confirms that the nuclei stained in H&E in the rotated cusp section are in fact nuclei. This same confirmation cannot be said for the bioreactor conditioned cusp section; the DAPI shows few nuclei retained within the cusp. There is over-staining seen in both the H&E and IHC for Vimentin of the bioreactor conditioned cusp that can be very misleading, but this has been recognized and noted in the analysis of the histological images.

IHC analysis suggests that the cells within the cusp of the rotated valve are qVIC-like, expressing Vimentin strongly and little to no true staining for α-SMA and prolyl-4-hydroxylase. Direct comparison of the image from a native cusp and the seeded/rotator conditioned cusp show few differences (figures 20 and 22). The lack of α-SMA expression in the conditioned cusps is encouraging because the goal is to achieve the quiescent-VIC phenotype for repopulation of the interstices of the cusp. Referring to Gimble et al.'s description of VIC phenotype, Section 2.4, the quiescent-VIC indicates normal, healthy interstitial cusp cells.

CHAPTER 6: CONCLUSIONS

In the tissue engineering of fully recellularized heart valves there is a need to establish a more clinically relevant interstitial population of cells and an improved, reproducible technique in which to do so. The present work developed an interstitial seeding technique by injection of cells directly into the heart valve cusp structure. The injection process was optimized and was shown to be a teachable and reproducible method. Through the analysis and comparison of rotational and bioreactor conditioning it was found that rotational conditioning of the recellularized valve is a better suited technique in which to obtain an implantable construct that more closely resembles that of a native heart valve. To support this conclusion we have shown the differentiation of hADSCs, in vitro, into a VIC-like phenotype that is found in the native aortic heart valve.

CHAPTER 7: RECOMMENDATIONS

7.1 Determining Cell Proliferation

The acellular aortic heart valve scaffold is believed to be a suitable micro-environment in which seeded cells can interact and embed themselves. The structure should encourage cell proliferation and remodeling of the scaffold. In order to determine whether or not the scaffold provides appropriate components and signaling molecules to promote cell proliferation it would be useful to stain histological sections for the Ki-67 Antigen. This particular nuclear protein is expressed during all active stages of the cell cycle (G_1, S, G_2, and M-phases), but is not present in resting cells (during G_0 phase) [46]. Positive expression would indicate that, in fact, over the course of the experiment cells had adapted to their micro-environment and the mechanical conditions to which they were exposed and began proliferating. This would lead to the confirmation of an appropriate cellular response while being conditioned in vitro.

7.2 Characterization of Material within Conditioned Cusps

It is important to completely characterize the material that was either created or built up in the cusp structure. It is hypothesized to be cellular material from apoptotic cells that simply could not be appropriately removed from the structure due to the absence of macrophages within the in vitro setup. Several assays can be completed in order to determine nuclear apoptosis of cells in the structure; those include but are not limited to: TUNEL, Annexin V, and Caspase-3. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end-labeling) indicates DNA fragmentation, Annexin V tags cells that express phosphatidylserine on their surface, and Caspase-3 detects the activation of caspase enzymes during apoptosis [47, 48]. Histological sections similar to those seen in figures 18-22 need to be evaluated with the assays above.

7.3 Injection Point Concerns

Although it has been proposed that the elastin-rich ventricularis is able to self-seal after injection, there may be a need for a more active approach of sealing to ensure that cells will not leak out of the cusp when the needle is removed. If this is desired, either BioGlue or some derivative, like 'BTglue' from Tedder et al., could be used to seal the point of injection [40]. These compounds combine percentages of bovine serum albumin and glutaraldehyde into an adhesive that is used in clinical practice and tissue engineering research. The adhesive could be applied directly onto the injection site as the needle is removed from the cusp. It has also been suggested that a very fine suture could be used to secure the holes made in the cusp during air and cellular injection. For this approach to be applicable a dissection microscope would need to be used to visualize the 0.21mm (O.D. of needle from Hamilton Company Product Specifications) puncture that the needle makes when inserted into the cusp. There is concern surrounding the mechanical function of the cusp after employing either of these alternatives; would the cusp still be able to bend and stretch as the valve opens and closes, would the adhesive stiffen the cusp, would the extra punctures created by the suture cause too much damage, etc.

7.4 Long-Term In Vitro Studies Combining External and Interstitial Seeding Techniques with Rotational Period Followed by Bioreactor Conditioning

The experiments performed to develop this research project were only run for a maximum of 24 days with only interstitial seeding being performed. Future studies should attempt to seed the aortic heart valve cusps interstitially and externally then condition the construct over the course of 4-6 weeks; rotational conditioning should occur during the first 3-4 weeks (evaluating at least one valve after this time period); followed by bioreactor conditioning for the remaining 2-3 weeks.

The final conditioned valves would be compared to static controls, post-rotational conditioning, and the studies performed previously.

7.5 Ovine Model In Vivo Studies

Preliminarily speaking, the cells that were seen in the valves after rotational conditioning seem to be an appropriate representation of physiologically relevant quiescent valvular interstitial cell type (positive for Vimentin and negative for α-SMA and P4HA3). If the hypothesis that the excess material in the cusp is apoptotic cellular matter, then the only way to clear it out would be to introduce the construct to a macrophage containing environment by implanting the conditioned construct into an animal model. This in vivo model would serve to test if the interstitial cells continue to display qVIC-like phenotype and if the cusp structure can indeed remodel under physiological conditions. Valves would be implanted into juvenile sheep in the pulmonary position; 6 months and one year time points would be evaluated.

7.6 Clinical Translation

Many of the current tissue engineering strategies rely on extensive seeding and conditioning protocols that cannot be easily translated into the clinical setting. To reiterate from previous statements, many researchers use cell types that are difficult, extremely painful, or even impossible to harvest from a living person. A recent article on the clinical translation of bioreactor systems into operating rooms aimed to provide patients with an autologous, partially recellularized valve replacement within 2-3 days of harvesting cells [21]. Although the group has not completely been able to develop all aspects of the protocol thus far; they have come up with what seems like a reasonable procedure and timeline for tissue engineering valve replacement to be implemented in the clinical setting.

The technique of cell harvesting, seeding, and conditioning that is employed in this research could be readily manipulated to provide a clinically relevant treatment for patients needing a heart valve replacement. Within the clinic, adipose tissue can be harvested quickly and with substantially less pain/discomfort relative to other methods of cell collection. From the lipoaspirate collected, adipose-derived stem cells can be isolated and plated. As discussed in section 2.3, a larger yield of stem cells is possible when using ADSCs as opposed to most other cell sources. This is a very important aspect in obtaining a sufficient amount of cells with which to seed the valve construct. The cells can then be seeded within the cusp through the injection technique shown in figure 11. The seeded valve can immediately be subjected to rotational conditioning. The cells within the valve can be maintained with simple media changes every 3-4 days. The rotating device is small and easily operated. It can also be redesigned to fit into a small incubator so it is not an over cumbersome system. The longer the patient is able to wait for the replacement, the longer the cells will have to secure themselves in the cusp structure, but if necessary the construct could be implanted within several days of seeding.

APPENDICES

Appendix A: Design criteria for tissue engineered heart valves [19]

Table 2 Design criteria for tissue engineered heart valves

Parameter	Conventional (mechanical, bioprosthetic)	Tissue engineered
Closure of leaflets	Rapid and complete	Rapid and complete
Fluidic function	Good	Potentially identical to native valve in terms of effective orifice area and pulmonary and systemic pressure and flow levels
Risk of thrombosis	Yes (especially high in mechanical valves which require anticoagulation causing vulnerability to hemorrhage)	Endothelial surface should inhibit thrombogenesis
Surgical insertion	Easy and permanent	Easy and permanent
Risk of structural dysfunction	Degradation of materials, which are rare in mechanical valves but high in bioprosthetic valves from tissue degredation and calcification	Potentially resistant to tissue degradation and calcification owing to tissue viability
	Rare in mechanical valves	
	High in bioprosthetic valves from tissue degradation and calcification.	
Risk of infection	Ever present	Resistant
Cellular function	None	Physiological VIC and VEC function
Tissue function	Durable and stable, chemically inert, nonhemolytic	Durable and stable
		Functional anatomic characteristics: collagen, elastin, GAG distribution and structure
		Functional mechanical characteristics: anisotropy, high tensile strength, low effective flexural rigidity
		Remodel according to need
		Somatic growth
Geometry	Set by design (mechanical/pericardial bioprosthetic) or tissue source (porcine bioprosthetic)	Designed at time of implantbased patent-specific geometry
		Somatic growth adaption may require/allow for nonuniform changes
Surgical considerations	Sterility, design, and implantation procedures well established	Sterility
		Practical considerations in cell sourcing
		Implantation
		Suturing/physical handling
In vivo monitoring requirements	Standard hemodynamic function	Hemodynamic conditions over initial and long-term remodeling periods
	Degeneration/regurgitation	Cellular physiological and phenotype, mass and content levels
		Changes in tissue components and mass changes
		Structure and mechanical properties
		Differences between neonate, juvenile, and adult

Appendix B: Major challenges in clinical translation of heart valve tissue engineering [16]

Table 1

Major challenges in clinical translation of heart valve tissue engineering

Challenges	Research directions
TEHV components and their interactions are complex, heterogeneous and dynamic	Define cell/scaffold/bioreactor combinations that optimize construct composition and properties (*in vitro*)
Correlation of *in vitro* generated construct structure and properties with *in vivo* outcomes has not been demonstrated	Determine and validate correlations between *in vitro* conditions, elements, structure, properties and *in vivo* function
Quality control of construct structure and function is likely to be difficult	Develop guidelines, tools and metrics for the pre-implantation characterization of TEHV structure, function and quality
Animal models may not reliably predict human outcomes	Develop and validate animal models that will test key biological processes and correlate with human outcomes
TEHV structure is likely to be evolving *in vivo* and ongoing function may be less predictable than with conventional valve replacement technology	Develop guidelines, tools and metrics for the *in vivo* characterization of dynamic TEHV structure, function and quality
TEHV function will depend upon patient response to implantation and integration with the recipient's tissues more than with conventional valve replacement, and individual patient responses may be highly variable	Identify/validate biomarkers both predictive of and assess patient variability in implant success/failure and capable of non-invasive *in vivo* monitoring and potential control
Remodeling processes after implantation may release or change seeded cells and recruit host cells	Develop tools to monitor the fate of transplanted and endogenous cells (location, function, viability, phenotype)
Regulatory processes and approaches are not yet well established	Create suitable regulatory approaches to engineered tissue valves that will ensure safety and efficacy

TEHV = Tissue Engineered Heart Valve.

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